NAM, HEESUN, Ph.D. Novel Mechanisms Regulating Inflammatory Gene Expression in Adipocytes. (2012)
Directed by Dr. Ron F. Morrison. 161pp.

Obesity and diabetes are major public health concerns worldwide that contribute to cardiovascular disease, hypertension, and stroke. Research over the last two decades has revealed an important role for adipose tissue (AT) in the regulation and control of whole-body metabolic homeostasis and that chronic AT inflammation is an important pathogenic mechanism that links obesity and diabetes. While it is now recognized that inflammation is increased in AT with obesity, studies are underway to identify key inflammatory mediators and their downstream pathways that contribute to adipocyte inflammation. Therefore, data presented in this dissertation demonstrate that three novel mechanisms that may mediate obesity-induced inflammation in adipocytes.

Interleukin (IL)-12 family cytokines are heterodimeric proteins mostly expressed in classic immune cells and play critical roles in innate and adaptive immunity. However, recent evidence has shown that plasma levels of IL-12 family cytokines are elevated in obesity and diabetes, yet little has addressed a role for IL-12 family cytokines in adipocytes under any condition. Data presented in this dissertation demonstrate that various IL-12 family members are highly induced in AT under conditions of genetic and diet-induced obesity that is associated with increased inflammation and IR. Subsequently, we show that both preadipocytes (PAs) and adipocytes (ADs) secrete IL-27 in response to inflammatory stress and demonstrate a novel function of IL-27 in adipocytes that is dependent on environmental cues.

While PAs and ADs both secrete IL-27 protein, divergent mRNA regulation of the IL-27 subunits (i.e., p28 and EBI3) was observed in response to tumor necrosis factor α

(TNFα) in adipocytes. As our data indicated a role for epigenetic modifications in differential IL-27 mRNA expression, we further determined that histone deacetylases (HDACs) play a key role in TNFα-induced p28 gene expression. Thus, these data demonstrate that epigenetic modification of histones potentially regulates the divergent outcomes observed with p28 and EBI3 gene expression during inflammatory stress.

While extensive studies have identified TNFα as a master cytokine involved in AT inflammation, questions regarding its inflammatory actions on gene expression remain unclear. Emerging evidence highlights an important role for the Golgi apparatus (GA) in TNFα-induced inflammation, in part, through TNF receptor (TNFR) storage and secretion to the cell-surface, thus allowing for TNFα signaling. Our findings demonstrate a critical role for the GA in TNFR cell-surface expression and TNFα signaling as well as suggest a novel mechanism for the GA in TNFα-induced inflammation that potentially involves Golgi-localized co-factor(s) necessary for transcriptional gene expression. Collectively, data presented in this dissertation provide seminal evidence for novel mechanisms regulating adipocyte inflammation, potentially linking obesity with metabolic diseases.

NOVEL MECHANISMS REGULATING INFLAMMATORY GENE EXPRESSION IN ADIPOCYTES

by

Heesun Nam

A Dissertation Submitted to
the Faculty of the Graduate School at
The University of North Carolina at Greensboro
in Partial Fulfillment
of the Requirements of the Degree
Doctor of Philosphy

Greensboro
2012

Approved by

_____
Committee Chair

In dedication to my family, for your continued support and love.

APPROVAL PAGE

This dissertation has been approved by the following committee of the Faculty of the Graduate School at The University of North Carolina at Greensboro.

Committee Chair _____

Committee Members _____

_____

_____

_____
Date of Acceptance by Committee

_____
Date of Final Oral Examination

ACKNOWLEDGEMENTS

First of all, I would like to gratefully and sincerely thank my mentor Dr. Ron Morrison. It has been an honor and privilege to work with him. His excellence guidance, understanding, patience, and most importantly, his friendship were paramount in providing a well-rounded experience consistent my long-term career goals. He encouraged me to not only grow as a scientist and critical thinker but also as a better person. I would like to express my deepest gratitude to Dr. Morrison for the opportunity to work in his lab.

I would also like to thank all of my committee members: Dr. George Loo for his constant support and encouragement during my graduate career, Dr. Lynda Brown for being a wonderful teacher and for encouraging and supporting me throughout my graduate career, and Dr. Karen Katula for great support, advice, and willingness to spend time to talk with me. All my committee members have been devoted and given wonderful guidance to make this dissertation possible.

In addition, I would like to thank all of the members of the Morrison Lab, past and present. Specially, Brad and Dr. Robin Hopkins, thank you for your tremendous help and many thoughtful conversations and advice. They are willing to help me with not only understanding science, but also many ways in life. Thank you for all friendship and support.

I would like to extend my appreciation to the faculty, staff, and fellow graduate students, both past and present, in the Department of Nutrition. Their support and assistance made my graduate life extremely comfort and special. It has been memorable experience to me. I would also like to acknowledge the Department and the Graduate

school for the funding I have received. Also, I would like to acknowledge external funding I received from Wrigley Research Institute for ASN Predoctoral Fellowship.

Most importantly, I would like to special thanks to my family for their unconditional love, support, and encouragement. I would especially like to acknowledge my parents for their love, patience, and support. I am truly blessed to have them by my side. I would also like to acknowledge my in-laws for their support and love. To my best friend and love of my life, Sooeun, there is no word to express how much I appreciate all your love, understanding, and support. I cannot ask for more from you. Every day I think how much I am blessed to have you in my life. You are my inspiration and motivation. Without you, I would not be where I am today.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER I

INTRODUCTION

Significance of Research

Obesity and diabetes mellitus are major public health concerns that globally afflict millions of individuals and subsequently lead to cardiovascular disease, hypertension, stroke, and neuropathies. The prevalence of obesity and diabetes has increased worldwide to 1.6 billion and is projected to double by 2030, highlighting an urgent need to delineate the molecular mechanisms that link these diseases and, therefore, allow for the development of better therapeutical strategies. Experimental and clinical evidence has established that chronic, low-grade inflammation links obesity to insulin resistance (IR) and ultimately diabetes. This is readily evident in preclinical animal models of obesity where genetic and pharmacological attenuation of pro-inflammatory cytokines and chemokines improved insulin signaling. Moreover, these studies highlight adipose tissue (AT) as a critical regulator involved in the secretion of adipocyte-derived cytokines and chemokines (i.e., adipokines), implicating AT in the direct regulation of obesity-induced IR. However, key mechanisms mediating obesity-induced inflammation in AT remain unresolved. Tumor necrosis factor (TNF) α is a pluripotent cytokine that mediates multiple inflammatory processes and immune responses. It has been clearly demonstrated that increased TNFα induces inflammatory gene expression and IR in AT during development of obesity. However, underlying mechanisms involving regulation of TNFα-induced inflammatory cascades in adipocytes are still unclear. As AT inflammation plays a central role in obesity-induced IR and

diabetes, the proposed research is significant as it establishes three novel mechanisms regulating adipocyte inflammatory gene expression. The objectives of this dissertation are to 1) characterize obesity-induced regulation of Interleukin (IL)-12 family cytokines in insulin-responsive tissues and cultured adipocytes, 2) elucidate novel mechanisms regulating IL-27 by histone deacetylases under conditions of inflammatory stress, and 3) investigate a novel role of the Golgi apparatus in mediating TNFα-induced inflammatory gene expression in adipocytes. The literature relevant to this research project is reviewed below.

Review of Literature

Obesity and diabetes. The prevalence of obesity has risen sharply in the United States in recent years. Among adults aged 20 years or older, 33.9 % are clinically obese, (1) as defined by a body mass index (BMI) of 30 kg/m^2 or above. The sharp rise in obesity has been linked to increased type 2 diabetes (T2D) and cardiovascular disease, leading to public health concerns and plaguing an already overburdened health care system with costs approaching $140 billion annually (2,3). While the onset and progression of obesity is influenced by genetic, social, cultural, and environmental factors, it is generally recognized that excessive energy consumption and a sedentary lifestyle directly contribute to excess AT mass (4). Furthermore, it is widely accepted that increased fat accumulation in humans and animals correlates directly with the development of insulin resistance (IR) and ensuring diabetes. Indeed, weight loss therapy in obese adults was shown to concurrently improve pancreatic β-cell function, decrease plasma glucagon concentrations, and improve insulin action (5). In addition,

weight loss through exercise has been shown to enhanced fat oxidation and improved insulin sensitivity in obese adults (6).

The World Health Organization (WHO) estimates that more than 1.1 million people die each year because of diabetes (7). More alarming, death due to diabetes is expected to grow, as prevalence rates have risen to 171 million and are projected to double by 2030 (8). Concomitant with these statistics, prevalence estimates from the National Health and Nutrition Examination Survey (NHANES) concluded that approximately 26 million U.S. adults are diabetic (i.e., fasting blood glucose (FBG) > 126 mg/dL) and another estimated 79 million pre-diabetic (i.e., FBG of 100-125 mg/dL). Similar to obesity, increased prevalence of diabetes contributes to increased cardiovascular disease, stroke, kidney failure, and amputation. These diabetes-linked co-morbidities add burden to the health care system with costs reaching $376 billion per year (9). As diabetes is coupled to the epidemic rise in obesity, it is not surprising that the number of adults and children diagnosed with diabetes continues to rise. Based on these statistics, increased knowledge concerning mechanisms regarding obesity-induced IR will become vital for disease prevention and treatment of future generations.

Adipose tissue as an endocrine organ. Obesity is characterized by expansion of AT mass. AT is a complex, highly innervated organ primarily composed of adipocytes and determined, but undifferentiated preadipocytes as well as fibroblasts, nerve cells, endothelial cells, smooth muscle cells, macrophages, and lymphocytes. Together, these diverse cell types in AT function as an integrated unit that dynamically regulates energy homeostasis. Historically, AT was regarded simply as a storage depot for excess energy during periods of caloric abundance. Research from recent years has determined that

AT has a much more complex and dynamic function as it acts as an endocrine organ secreting various factors into the blood. In 1987, the endocrine role of AT was first introduced, where it was observed that adipsin was secreted from adipocytes (10,11). The subsequent identification and characterization of leptin, an adipose-derived hormone regulating whole-body energy homeostasis, firmly established AT as an endocrine organ (12,13). It is now clear that AT secretes a plethora of peptide hormones, cytokines, or non-peptide biologically active molecules, which play a critical role in vascular homeostasis, immunity, and energy metabolism in tissues such as liver, muscle, and heart. While several key factors have been suggested to alter AT function and trigger IR, the onset of inflammatory gene expression has clearly been identified as a critical event coupling obesity to IR.

Obesity, inflammation, and insulin resistance. It is now well-established that chronic, low-grade inflammation within AT plays a key role in linking obesity to IR and subsequently T2D (14). Several investigators have shown that chemokine and cytokine levels are increased during obesity, while ablation of these pro-inflammatory molecules improves insulin signaling. What triggers AT inflammation remains a heated topic of debate; however, recent studies suggest that endoplasmic reticulum (ER) stress is crucial for the initiation and integration of inflammatory pathways. The ER plays a critical role in protein folding, maturation, storage, transport, and lipid metabolism (15). It has been suggested that obesity over-exerts ER actions and, therefore, uncouples metabolic homeostasis within the adipocyte leading to IR. Accordingly, it has been shown that nutrient excess (e.g., glucose and free fatty acids) leads to ER dysregulation, thus initiating and subsequently activating inflammatory signaling cascades mitogen-activated

protein kinases (MAPKs), particularly c-Jun N terminal kinase (JNK), and nuclear factor kappaB (NF-κB) that promote inflammation (16-18).

Oxidative stress is a second mechanism implicated in the initiation of inflammation during obesity. Nutrient overload in AT has been shown to disrupt mitochondrial function leading to over-production of reactive oxygen species (ROS). Elevated levels of ROS impose oxidative damage and activate inflammatory signaling cascades within adipocytes. In addition to mitochondrial dysfunction, increased fat accumulation and BMI are closely correlated with increased systemic oxidative stress (19-21). Thus, it is not surprising that studies link increased ROS production during obesity to the activation of MAPKs and NF-κB and subsequently IR. Moreover, hyperglycemic conditions derived, in part, from decreased insulin sensitivity further exacerbate ROS production in adipocytes enhancing pro-inflammatory conditions (22).

Taken together, ER and oxidative stress, alone or in combination, promote chronic inflammation through the activation of inflammatory signaling pathways, in particular JNK and NF-κB. It is well established that both signaling pathways increase the production of pro-inflammatory cytokines in adipocytes, including tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and monocyte chemoattractant protein-1 (MCP-1) (23). Activation of inflammatory signaling pathways within the adipocyte leads to enhanced AT inflammation through paracrine and endocrine actions of secretory proteins such as MCP-1 (24-26). MCP-1 functions predominantly as a chemoattractant protein highly specific for monocyte and macrophage recruitment to AT during obesity (Fig.1.1). Ligand binding of MCP-1 to the MCP-1 receptor (i.e., CCR2) on macrophages promotes macrophage chemotaxis to AT as well as the subsequent activation and

secretion of pro-inflammatory cytokines including TNFα into the local milieu (27,28). Therefore, adipocyte dysfunction and macrophage accumulation play an important role in regulating AT inflammation that contributes to obesity-induced IR (Fig.1.1).

However, the plethora of inflammatory factors, that increase during obesity and regulate AT inflammation and IR, remain unresolved. It has been suggested that immunity and metabolism co-developed as functional relationships closely shared by adipocytes and macrophages (29-31). For example, both adipocytes and macrophages can be activated by pathogens (e.g., lipopolysaccharide) and secrete cytokines with pro- and anti-inflammatory properties (32). Moreover, the initiation and maintenance of immunity is metabolically demanding and cannot operate efficiently during conditions of energy deficit (33). Thus, the evolutionary pressures to survive would have favored energy efficiency and storage for times of food deprivation. Antithetically, co-evolutionary development is thought to drive chronic, low-grade inflammation during modern periods of human existence where nutrient excess is high. This is clearly evident in AT of obese individuals, where levels of pro-inflammatory cytokine, TNFα, are elevated under conditions of energy overload (33,34). As genetic or pharmacological ablation of this cytokine blunts chronic low-grade inflammation, it has been suggested that TNFα play a critical role in the development of obesity-induced IR and progression to T2D.

Adipocyte and immuno-modulatory cell interactions. Recent evidence demonstrates a prominent role for different immune cells in the regulation of AT inflammation and the development of IR in the pathophysiology of obesity. In addition to macrophages and monocytes, natural killer (NK) cells can infiltrate AT (35-37). Moreover, recent data demonstrate that various lymphocytes, including T regulatory

(Treg), T helper (Th), and T effector (Teff) cells, as well as B cells localize in AT depending on the stage of obesity (36,38,39). Indeed, studies suggest that T lymphocytes are present in AT even before macrophage infiltration, and that this early infiltration activates inflammatory signaling pathways that drive production of pro-inflammatory chemokines like MCP-1, thus stimulating macrophage accumulation (36). These observations suggest that cross-talk between different immuno-modulatory cell types play a critical role in the development of AT inflammation and IR (40,41). Collectively, these studies demonstrate that cytokines and chemotactic mediators from multiple cell types work in concert with infiltrating immune cells to drive and exacerbate the inflammatory process in AT.

IL-12 family cytokines. While it is now well recognized that an array of inflammatory cytokines and chemokines (e.g., TNFα, IL-6, and MCP-1) are increased in AT with obesity, extensive studies are underway to identify other inflammatory mediators that contribute to inflammation and chronic metabolic diseases like T2D. IL-12 family cytokines are heterodimeric proteins that are predominantly expressed in antigen-presenting cells and play central a role in innate and adaptive immunity (42). IL-12 family cytokines respond to pathogens and activate cell-mediated antigen-specific defense mechanisms, which include increased expression of cytokines (43). IL-12 family cytokines include IL-12, IL-23, IL-27, and the recently identified IL-35. Each cytokine is composed of an α chain (i.e., p35, p19, and p28) and a β chain (i.e., p40 and Epstein-Barr virus-induced gene (EBI) 3) that dimerize and signal through unique heterodimeric receptors composed of IL-12Rβ1, IL-12Rβ2, IL-23R, gp130, and WSX-1 (44) (Fig.2.1). IL-12 family cytokines share structural homology to IL-6. The α chain is homologous to other single-chain cytokines, such as IL-6, whereas the β chain shares homology to the

soluble form of IL-6Rα chain, suggesting that IL-12 family cytokines evolved from a member of the IL-6 cytokine superfamily. In keeping, IL-12 family cytokines are now considered part of the IL-6 superfamily (45,46). While pairing between IL-12 cytokine subunits and receptors is conserved, the origin, activity, and kinetic expression are cell-type and condition specific. Similar to other pro-inflammatory cytokines, the production of IL-12 family cytokines is controlled by positive and negative regulatory elements. However, expression of both gene subunits (i.e., α and β chains) within the same cell is thought to be necessary for secretion of a biologically active cytokine (47,48).

The first member of the IL-12 cytokine family that was identified was IL-12. IL-12 is composed of two disulfide-linked subunits p35 and p40 and is predominantly secreted from phagocytes, including macrophages, monocytes, neutrophils, and dendritic cells (DCs). These cell types play a critical role in immuno-regulatory actions, particularly through interferon (IFN) γ production and Th1 responses (46). In vitro and in vivo studies have shown that p40 can be secreted as a free monomer or homodimer (49,50), and that this IL-12p40 monomer/homodimer is not biologically active, but instead acts as an antagonist to the IL-12 heterodimer. Intriguingly, monomer/homodimer antagonism has only been demonstrated in mice and does not occur in humans (49). IL-12 signaling occurs when the IL-12 heterodimer binds IL-12Rβ1 and IL-12Rβ2. Receptor binding triggers transphosphorylation and subsequent activation of the Janus Kinase (JAK)/Signal Transducer and Activator of Transcription (STAT) pathway that ultimately results in phosphorylation of cytosolic and nuclear events involved in inflammation.

While IL-12 is composed of the p35 (α chain) and p40 (β chain) subunit, the p40 chain can also dimerize with p19 to form IL-23 (51). The discovery of IL-23

demonstrated a chain sharing characteristic that has since been described for the other IL-12 family cytokines. IL-23 binds IL-12Rβ1 and IL-23R, thus stimulating the JAK/STAT signaling pathway (43,51). IL-23 signaling through the JAK/STAT pathway drives IL-17 expression that in turn promotes the Th 17 cellular response. The third member identified in the IL-12 family is IL-27, which is composed of p28 (α chain) and EBI3 (β chain) (52). Finally, the most recently identified member of IL-12 family is IL-35. IL-35 is composed of p35 and EBI3 (53). IL-35 is mainly produced in Treg cells and exerts suppressive effects on T cell proliferation. Receptors for IL-35 remain unclear; although it has been hypothesized that IL-12Rβ2 and WSX-1 or gp130 serve as receptors for IL-35. While these cytokines share structural properties, divergent functions have been described for IL-12 family cytokines in the promotion of the immune response (44,54).

IL-27. As mentioned above, IL-27 is composed of p28 and EBI3. While most studies demonstrate that IL-27 secretion is restricted to myeloid cells including monocytes, monocyte-derived DCs, and macrophages (55), recent studies indicate that IL-27 is also regulated in non-immune cells. For example, EBI3 and p28 were expressed in syncytio-trophoblasts and extravillous trophoblasts throughout human pregnancy (56). Moreover, p28 and EBI3 were highly expressed in regions of vascular smooth muscle cells and endothelial cells in response to pro-inflammatory stimuli (e.g., TNFα and IFNγ). These studies further demonstrate that regulation of IL-27 is NF-κB dependent, as IL-27 expression was attenuated in the presence of the peroxisome-proliferator activated receptor (PPAR)γ agonist, rosiglitazone that blocked NF-κB binding to regulatory elements within the *EBI3* gene (57). Subsequent studies support a key role for NF-κB in the regulation IL-27. This is apparent in conventional DCs exposed to docosahexaenoic acid (DHA), where lipopolysaccharide (LPS)-induced expression of IL-12 family

cytokines including IL-27 was suppressed through NF-κB inhibition (58). Additionally, studies using NF-κB-deficient mice clearly demonstrate a role for NF-κB as the predominant regulator of inducible p28 and EBI3 gene expression in classical immune cells (55,59). However, the transcriptional regulation of IL-27 in non-immune cells remains largely unknown.

IL-27 is a unique IL-12 family member as reports have discerned two distinct functions in its regulation of the immune response: one as an initiator and the other as an attenuator of immune/inflammatory responses. IL-27 is a key cytokine that drives naïve T cells into the Th 1 subset at the initial step of differentiation. Upon differentiation, IL-27 actions reverse and subsequently inhibit activated Th cells. It is unclear why and how IL-27 exerts pro- and anti-inflammatory actions, however, recent studies demonstrate that differential IL-27-mediated regulation of STAT signaling pathways is essential for these contradictory actions (60). IL-27 binds to its heterodimeric receptor composed of gp130 and WSX-1, also known as T cell cytokine receptor (TCCR) (53). IL-6 family cytokines (i.e., gp130 cytokines) including IL-11, oncostatin M, leukemia inhibitory factor, cardiotrophin 1, and neuropoietin also use gp130 as a receptor component required for both ligand binding and signal transduction (61,62). Hence, IL-27 is also considered a gp130 cytokine that may share similar functions to that of IL-6.

IL-12 family, obesity, and inflammation. Recent evidence highlights the IL-12 family cytokines as prospective regulators linking obesity to IR. While plasma levels of select IL-12 family members are elevated with obesity, diabetes, and metabolic syndrome (63-66), their cellular origin has not been fully determined. Still, IL-12 family expression in response to nutrient overload and inflammatory signals in macrophages, a

major player in the development of AT inflammation, has been investigated. High levels of glucose (HG) have been shown to increase IL-12 gene and protein expression in mouse peritoneal macrophages (66). In addition, IL-12 mRNA expression is highly elevated in type 1 diabetic (T1D) mice and T2D mice compared to control littermates (64). These findings suggest that hyperglycemia promotes IL-12 gene and protein expression. Moreover, HG induces inflammatory signaling pathways including p38, JNK, and NF-κB in macrophages, where inhibition of these signaling pathways suppressed HG-induced IL-12 mRNA expression (66,67). Recent studies demonstrated that obese mice exhibited significantly increased mRNA levels of p40 in parallel with IL-6 and TNFα expression in epididymal AT (68,69). Although these studies suggest that AT can be an important source of IL-12 family cytokines under different conditions, their cellular origin has not been fully elucidated. Additionally, as evidence has established a mechanistic role for the gp130 cytokine in obesity-induced inflammation and IR (70-72), it has been hypothesized that IL-27, a recognized gp130 cytokine, potentially mediates obesity-induced IR, inflammation and T2D. These findings support a possible role for IL-12, IL-27, and other family members, as important mediators in obesity-induced AT inflammation linking excess nutrient intake to IR and diabetes. Finally, the functional relationships closely shared with adipocytes and macrophages, suggests that adipocytes produce IL-12 family cytokines in response to inflammatory stress, leading to the pathogenesis of obesity-induced inflammation and IR.

TNFα. As an active endocrine organ, AT secretes proinflammatory cytokines, chemokines, growth factors, and complement proteins called “adipokines,” that regulate systemic tissue metabolism, lipid metabolism and insulin sensitivity (73,74). As a result of the excess production of these pro-inflammatory adipokines, AT enters a state of

chronic inflammation that includes macrophage infiltration (75-78). The most noted and well-studied adipokine involved in obesity-induced inflammation and IR is TNFα. TNFα was originally identified as a mediator that caused necrosis of tumors in mice infected with endotoxin (79). Subsequent studies later reported that TNFα enhanced muscle wasting (cachexia) (80) as well as dyslipidemias (81). Today, TNFα is a well-defined cytokine that regulates multiple biological activities such as cell fate, immune function, cytokine production, and IR (82). TNFα is produced by a variety of cell types, including macrophages, lymphocytes, fibroblasts, and adipocytes in response to inflammation, infection, injury, and other environmental stresses (83).

TNFα is synthesized as a 26-kDa plasma membrane-associated monomer (mTNFα) that undergoes proteolytic cleavage by metalloprotease, TNFα converting enzyme (TACE) to produce a 17-kDa soluble TNFα (sTNFα) molecule (80,84). Both the membrane bound and soluble forms of TNFα have been shown to mediate cellular TNFα action through the binding of two distinct receptors, TNFα receptor (TNFR)-1 and TNFR2. Similar to sTNFα, the transmembrane form of both receptors can be released into circulation via proteolytic cleavage by TACE. Release of soluble TNFR into circulation is currently used as a marker for increased inflammation and disease pathology. TNFR1 and TNFR2 share homology in the extracellular TNF binding domains and have similar binding affinity for TNFα, yet studies support separate cytoplasmic actions for these receptors that consequently result in different biological outcomes (85-87). For instance, studies have shown that signaling from TNFR1 predominantly regulates inflammatory gene expression and IR in AT (87,88), while the consequences of TNFα-mediated TNFR2 signaling in AT remains unresolved. However, TNFR2 has been shown to increase the sensitivity of cells to TNFα as well as assist TNFR1 with

ligand binding through a process known as ‘ligand passing’, in which TNFR2 binds TNFα and passes bound TNFα to an open TNFR1 (89). However, the importance of ‘ligand passing’ in AT inflammation is not yet known.

TNFα and adipocyte. A role for TNFα in obesity-induced IR was first documented in the early 1990s. It was observed that obese diabetic rodents expressed elevated levels of TNFα compared to wild-type littermates, while neutralization of TNFα in obese animals improved insulin sensitivity (90). Since this initial report, elevated levels of TNFα have subsequently been shown in human obesity and directly correlated to increased adiposity and IR (91). TNFα can function in both an autocrine or paracrine manner within AT to promote inflammation. Moreover, some studies, while controversial, suggest an endocrine role for TNFα on systemic IR (92). Regardless of its endocrine role, evidence over the last 20 years clearly demonstrates that TNFα signaling plays a pivotal role in coupling obesity to IR in adipocytes (93,94). In addition, several downstream mechanisms have been clearly defined for TNFα signaling in the development of IR in adipocytes.

One mechanism by which TNFα impairs insulin sensitivity in adipocytes is through the regulation of cytosolic proteins that blunt insulin signaling and glucose uptake. Under normal physiologic conditions, insulin can bind to and activate its tyrosine kinase insulin receptor. Once activated, the insulin receptor phosphorylates select tyrosine residues on downstream targets including insulin receptor substrate (IRS) proteins (i.e., IRS-1, -2, -3, and -4) (95). Tyrosine phosphorylated IRS-1 activates the phosphatidylinositol 3-kinase (PI3K/AKT) pathway that stimulates glucose transporter 4 (GLUT4) translocation to the plasma membrane and, thus, increases glucose uptake

(92,96,97). TNFα interferes with these signaling events downstream of the insulin receptor leading to decreased insulin sensitivity. Most notably, TNFα-activated JNK and IKK phosphorylate IRS-1 serine residue (S307), which blunts activation of PI3K (98) and inhibits tyrosine phosphorylation leading to the dissociation of IRS proteins from the insulin receptor (99,100). Collectively, these events impair GLUT4 translocation and cellular glucose uptake.

While TNFα can inhibit insulin signaling through cytosolic actions, early reports demonstrated a regulatory role for TNFα on nuclear gene expression (14). For example, TNFα suppresses gene expression of many proteins required for insulin-regulated glucose uptake, such as IRS-1 and GLUT4 (101-103). Furthermore, TNFα down-regulates genes critical for adipocyte function and metabolism, such as CCAAT/enhancer binding protein α (C/EBPα) and PPARγ (92). Finally, TNFα represses genes that encode components of the electron transport chain. Collectively, the nuclear repression of these TNFα targets contribute to dedifferentiation of the adipocyte, increased circulating fatty acids, and mitochondrial dysfunction (104), subsequently leading to impaired insulin signaling and heightened AT inflammation. While TNFα can suppress critical genes involved in adipocyte function, it can also promote expression of genes involved in ER stress, oxidative stress, and inflammation further exacerbating conditions within the local milieu (102). A well-known example of TNFα-mediated gene expression in adipocytes involves the up-regulation and secretion of MCP-1 that contributes to macrophage infiltration, while ablation of MCP-1 attenuates AT inflammation and IR. Indeed, subsequent studies have since shown that pharmacological or genetic loss of these TNFα-induced genes improves inflammation and insulin signaling (105). Of note, TNFα can also induce TNFα as well as TNFR1 and

TNFR2 gene expression (106), demonstrating a feed forward mechanism of TNFα signaling that contributes to the state of chronic, low-grade inflammation.

TNFα signaling. Two major signaling pathways: MAPKs and NF-κB, sit at the nexus of TNFα action in both the nucleus and cytosol (26). Studies clearly highlight these two signaling pathways as instrumental in obesity-induced IR, as levels of MAPK and NF-κB activity are increased in vitro and in vivo and have been linked to impaired insulin sensitivity (100,107-109). More specifically, activation of these two signaling pathways is central to mediating actions of TNFα in the cytosol (i.e., serine phosphorylation of IRS-1) as well as the nucleus (i.e., adipocyte gene repression and inflammatory gene expression), thus promoting AT inflammation and IR (75,83,110).

MAPKs. MAPKs consist of a family of highly conserved serine/threonine (S/T) protein kinases that include extracellular signaling-regulated protein kinase (ERK), JNK, and p38 (Fig.1.2). Phosphorylation of both threonine and tyrosine residues of the (T-X-Y) motif within the activation loop by upstream kinases is essential and sufficient for MAPK activation. Once activated, MAPKs can phosphorylate target proteins and transcription factors within the cytosolic and nuclear compartments (111-113). Transcriptional targets of MAPK activity include heterodimers of *trans*-acting factors (e.g., c-fos, c-Jun, ATF2) that are collectively referred to as AP-1 transcription factors. It is generally recognized that ERK is activated by growth factors and mitogens and is associated with cell survival and that JNK and p38 are stress activated (e.g., pro-inflammatory cytokines, UV irradiation, and reactive oxygen species) and associated with cellular stress and apoptosis (114). However, biological processes regulated by MAPKs are cell-type dependent. The MAPK signaling pathway has been shown to be activated in AT in

response to TNFα stimulation and serve as mediators of insulin signaling through cytosolic phosphorylation of specific serine residues of IRS-1 as well as induction of gene expression involved in impaired insulin actions (26,111,115-117).

NF-κB. TNFα also signals through the activation of the NF-κB pathway (Fig.1.2). The NF-κB transcription factor family consists of five members, p50, p52, p65 (RelA), p62 (RelB), and c-Rel that form homodimers and/or heterodimers to regulate gene expression (118). NF-κB activation is canonically regarded as the primary pathway downstream of inflammatory stimuli that regulate cytokine and chemokine gene expression. Each NF-κB family member plays a unique role that cannot be functionally substituted by other members of the family (119-121), suggesting that distinct NF-κB family members can differentially regulate transcriptional events during inflammation. Inactive NF-κB complexes are sequestered in the cytoplasm by a family of inhibitory proteins known as inhibitors of NF-κB (IκBs). NF-κB activation by inflammatory stimuli is initiated by phosphorylation of IκBα by the IκB kinase (IKK) complex resulting in degradation of IκBs. NF-κB is released from IκBα to translocate into the nucleus where it acts as a transcription factor involved in both repression and induction of gene expression (118,122). In addition to IκBα degradation, evidence demonstrates that posttranslational modifications of NF-κB subunits are necessary for the complete activation of NF-κB. One example of this includes the NF-κB subunit p65, where phosphorylation and acetylation alter transcriptional activity of NF-κB (123-125). Once activated, nuclear NF-κB dimers bind κB sites within the distal and proximal promoter of target genes and regulate transcription through the recruitment of co-activators and co-repressors (126,127).

Co-regulators of NF-κB. Emerging evidence indicates that transcriptional activity of NF-κB is also regulated by transcriptional co-activators and co-repressors, which themselves can be targeted by multiple signal transduction pathways (126,127). Moreover, regulation of co-activators and co-repressors is cell-type and condition-specific (128,129), potentially providing a mechanism for divergent biological outcomes assigned to transcription factors within the same family. For example, RIP140 is a well-known transcriptional co-repressor of PPARs (130) that regulate glucose uptake and fatty acid oxidation in metabolically active tissues (131-134). Conversely, RIP140 can also act as a co-activator of inflammatory gene transcription in macrophages (135). Collectively, studies demonstrate that co-activator and co-repressor function is not universal and can act in an opposing manner depending on gene environments (136,137).

The co-activators of NF-κB include CREB-binding protein (CBP/p300) and steroid receptor coactivator (SRC) proteins. CBP/p300 and the SRC proteins possess intrinsic histone acetyltransferase (HAT) activity. Histone acetylation serves an epigenetic function by acetylating specific lysine residues of nucleosomal histone proteins to alter the electrostatic properties of chromatin in manner allowing for gene transcription (138). The co-repressors of NF-κB include silencing-mediator for retinoid and thyroid hormone receptor (SMRT), nuclear receptor co-repressor (NCoR), and histone deacetylases (HDACs) (139-141). SMRT and NCoR do not possess enzymatic activity, but can trigger the catalytic activity of HDACs. In contrast to histone acetylation, HDACs function to deacetylate nucleosomal histones and alter chromatin in a manner that leads to gene repression (142).

HDACs. Epigenetic control encompasses heritable changes in gene expression or cellular phenotype caused by mechanisms other than changes in the DNA sequence. Aberrant transcription due to epigenetic modification on gene expression has been clearly linked to diseases including cancer and metabolic diseases (143). Emerging evidence has demonstrated that posttranslational modification of histone proteins by acetylation or deacetylation is important epigenetic phenomena that play critical roles in the modulation of chromatin topology and the regulation of gene expression (144). HDACs activity historically involves removal of acetyl groups from the nucleosomal histone proteins, influencing nucleosome structure and altering gene transcription (145). While histone acetylation is often correlated with gene activation, HDACs act to silence genes by reversing regulatory acetylation of histone proteins (Fig.1.3). Eighteen HDACs have been identified and divided into four classes based on similarity to yeast transcriptional regulators (146) (Table1.1). As their name indicates, it has generally been suggested that the activity of HDACs is directed at histone protein. Conversely, evidence using *Drosophila* and the yeast indicated that HDAC activity can contribute to gene activation (147-150). In addition, recent studies have shown that HDAC inhibition attenuates gene expression, suggesting HDAC activity is critical in activating some genes (151-153).

It has been shown that many HDACs are at least partially cytoplasmic (154-157), and studies demonstrated their action on non-histone substrates, including transcription factors such as NF-κB (124). As it has been suggested that acetylation/deacetylation is also important regulation for signaling transduction and gene transcription, acting in a manner similar to phosphorylation and dephosphorylation (158), acetylation of NF-κB protein is required for transcriptional activation (123). p65, the subunit of NF-κB that

contains the transactivation domain, is acetylated by a general transcriptional coactivator, CBP, which possess intrinsic HAT activity, and the acetylation enhances the DNA binding activity of NF-κB (159,160) (Fig.1.4). Evidence apparently appears that HATs and HDACs target non-histone proteins throughout various cellular compartments, where estimates of approximately 3000 proteins are regulated by acetylation/deacetylation (161,162). Thus, it is not surprising that HDACs regulate NF-κB through deacetylation of regulatory elements within the NF-κB gene as well as modification of NF-κB protein (163,164). While central dogma suggests that HDACs lead to gene repression, others have shown opposing roles for HDACs on gene expression. Indeed, treatments with pan-HDAC inhibitors such as trichostatin A (TSA), which block all HDACs except class III, have been shown to both enhance and suppress gene expression (165). Not surprising then, HDAC inhibitors can promote NF-κB activity by increasing p65 acetylation (123,166,167) and diametrically act in an anti-inflammatory manner through the repression of inflammatory genes, demonstrating that HDAC activity is cell-type and environment specific (168-170).

HDAC inhibitors. As HDACs appear to play an important role in the regulation of gene expression, deregulation of HDACs is associated with a variety of pathophysiological processes, including cancer, neurodegeneration, and inflammation (144). Since the first HDAC inhibitor, SAHA (Zolinza) was approved by the FDA for the treatment of cutaneous T-cell lymphoma (171), extensive studies have been underway to develop of novel small molecule inhibitors of HDACs. The general property of most HDAC inhibitors is a structure consisting of a zinc-binding group in the active site, deacetylase domain (150). There are four chemical classes of HDAC inhibitors: hydroxamic acids, short chain fatty acids, cyclic peptides, and bezamides (Table1.2).

While, relative potencies and selectivity of these HDAC inhibitors vary between and within classes (172), hydroxamic acid (i.e., TSA), which contains the greater zinc-chelating properties, is recognized as a potent (i.e., nanomolar concentrations) pan-HDAC inhibitor. In contrast, the short chain fatty acids are relatively less potent (i.e., milimolar concentrations) HDAC inhibitors as they provide modest selectivity towards class I HDACs. Cyclic peptides and benzamide HDAC inhibitors are generally highly selective for HDAC1, 2, and 3 (173). The class III HDACs, also known as sirtuins are NAD+ dependent HDACs also shown to play important roles in transcriptional regulation (174). Moreover, a few molecules have been discovered as inhibitor of sirtuins, including the vitamin, nicotinamide (175). While HDACs appear to be highly involved in the regulation of inflammatory gene expression, it is still unclear what role HDACs play in the transcriptional activation of inflammatory gene expression in adipocytes.

TNFα and Golgi Apparatus. Although TNFα can bind and signal through TNFR2, most of its activity regarding inflammatory gene expression and IR occurs through TNFR1 where ligand binding leads to internalization of the ligand-receptor complex (176,177). While some TNFα mediated-events depend on internalization, others are terminated as the receptor is recycled through recycling endosomes back to the membrane with degradation of the ligand by lysosomal activity (178). If not recycled or replaced, the outcome of internalization would be a decrease in surface receptor density leading to a decrease in TNFα sensitivity. A similar loss in TNFR on the surface occurs following enzymatic cleavage of membrane bounded receptors by TACE resulting in truncated, non-functional, soluble receptors (i.e., sTNFR) that are known to play a role in dampening TNFα activity (179,180). Thus, vesicular trafficking of TNFR, either from the de novo synthesis in the ER or through recycling endosome is critical for

TNFα action. To this point, others have also reported that TNFR1 is predominantly localized to Golgi apparatus (GA) in a perinuclear compartment as opposed to cell surface expression providing a means to curtail receptor sensitivity when TNFα action is not warranted. As this pool of TNFR1 has been shown to translocate to the surface upon TNFα stimulation, it has also been suggested that the Golgi pool serves a reservoir to increase cell surface receptor density when TNFα action is warranted (181). It has also been demonstrated that the cytoplasmic domain of the TNFR1 possess a Golgi-localization sequence (182,183), but how and under what conditions this localized trafficking occurs has yet to be determined. Furthermore, to date, Golgi-associated TNFR1 has been reported for only a few cell types suggesting the possibility of a cell-type, condition-specific phenomenon (181,182,184). As studies have clearly demonstrated the dynamic nature of TNFR1 surface expression influenced by receptor internalization as well as receptor shedding, vesicular trafficking, which may or may not involve Golgi activity, represents an important regulatory mechanism mediating TNFα action. Despite the importance of TNFα signaling in the etiology of obesity-induced IR, no information regarding a role for the GA in regulating TNFα signaling in adipocytes has emerged.

The Golgi Apparatus. The Golgi is a perinuclear organelle that modifies and packages proteins in route to their final destination within the cell. While many plasma membrane proteins (e.g., receptors) are modified in the Golgi, this organelle plays a critical role of packaging secretory proteins (e.g., ligands, cytokines) into vesicles prior to their transport to the cell surface for exocytosis. The Golgi organelle (commonly referred to as the ‘Golgi apparatus’) is comprised of stacks of membrane-bound structures known as ‘cisternae’ where each cisterna contains enzymes that modify and package proteins

that move through the organelle (Fig.1.5). The cisternae are arranged by structural and functional significance where newly synthesize proteins leaving the ER enter the Golgi at the *cis*-Golgi network (CGN), oriented in space nearest to the nucleus, and exit the *trans*-Golgi network (TGN) on the opposite face of the organelle in route to their final destination.

Transport of protein cargo between different regions of the GA as well as between many organelles (e.g., ER) and structures (e.g., plasma membrane) involves 'budding' of vesicles from the donor compartment (185,186). Formation of each vesicle is directed by a protein coat that forms a structural lattice that is critical for the budding process to occur. Each protein coat is specific for the region in which the vesicle is formed as well as its final destination, thereby playing a directing role in vesicular trafficking. COP-II proteins mediate vesicle budding of ER membranes and direct cargo export from the ER to the GA. Anterograde transport through the GA (i.e., away from the CGN) as well as retrograde transport (i.e., toward the CGN) is directed by COP-I protein coated vesicles. Transport from the TGN to final destination is directed by clathrin-coated vesicles where clathrin is recruited by the adapter protein, AP-1, which is different from clathrin-coated vesicles originating from other donor membranes (e.g., plasma membrane) where the clathrin is recruited by different adaptor proteins.

The coating of each vesicle with lattice proteins depends on small GTP-binding proteins such as Sar1 that initiates recruitment of COP-II proteins to ER vesicles and ADP-ribosylation factors (ARFs) that regulated COP-I recruitment to GA vesicles (187,188). ARFs cycle between active GTP-bound states and inactive GDP-bound states through GDP-GTP exchange mediated by guanine exchange factors (GEFs). The

ARF-GEFs, BIG1 and BIG2 are potently and reversibly inhibited by the fungal metabolite, brefeldin A (BFA) with a rare degree of specificity (189,190). At nanomolar concentrations, BFA induces the release and dispersal of all COP-I proteins from GA vesicles (Fig.1.5) as well as clathrin proteins that utilize specific adaptors (e.g., AP-1) leading to a collapse of the CGN to the ER and merging of the TGN with the microtubule organizing center. Following 20 years of investigation, there is strong consensus that the molecular targets of BFA are mostly confined to these GEFs where exposure to BFA at nanomolar concentrations results in rapid (< 2 mins) and reversible dispersal of GA coat proteins, thereby inhibiting GA functions (e.g., secretion) without affecting COP-II vesicle formation from the ER as well as clathrin-coated vesicles important for endosomal trafficking as well endocytosis or internalization receptors at the plasma membrane.

Review Summary. Obesity and T2D are major public health concerns worldwide that contribute to cardiovascular disease, hypertension, and stroke. Research over the last two decades has revealed an important role for AT in the regulation and control of whole-body metabolic homeostasis and that chronic AT inflammation is an important pathogenic mechanism that links obesity and diabetes (2,25,191). Early evidence demonstrated a role for inflammation where it was discovered that TNFα was overproduced in AT of obese mice. TNFα is a major cytokine regulating AT function and metabolism by activating multiple signaling pathways in order to activate expression of a number of pro-inflammatory genes. Accumulation of macrophages and other immuno-modulatory cells within AT along with adipokine secretion drives AT inflammation ultimately resulting in delipidation and systemic IR (14,23,192). While it is now recognized that inflammatory cytokines and chemokines are increased in AT with obesity, extensive studies are underway to identify other inflammatory mediators or

signaling pathways that contribute to adipocyte inflammation and IR. IL-12 family cytokines have emerged as a new mediators involved in obesity-related inflammatory diseases. Plasma levels of several IL-12 family cytokines have been shown to be up-regulated in obesity and diabetes (63-65), yet limited studies have delineated the regulation and role for these molecules in the adipocyte under conditions of inflammatory stress. While others evidence has demonstrated a role that HDACs in regulating inflammatory gene expression, there are few reports addressing their role regarding inflammatory gene expression in adipocytes. Albeit, extensive studies have identified TNFα as a master cytokine involved in AT inflammation, questions regarding its inflammatory actions on gene expression remain unclear. Therefore, we proposed the following study objectives to elucidate novel mechanisms regulating inflammatory gene expression in adipocytes, potentially linking obesity to inflammatory diseases.

Study Objectives

Chapter II. Inflammation is an important pathogenic mediator linking obesity and diabetes. Recent evidence highlights IL-12 family cytokines as prospective inflammatory mediators linking obesity to IR. However, little is known about regulation of this family cytokine with regards to obesity in insulin responsive tissues. Therefore, the objective of the study summarized in Chapter II is to examine the regulation of IL-12 family cytokines and receptors in insulin responsive tissues of genetic and diet-induced obese mouse models as well as cultured undifferentiated preadipocytes and mature adipocytes. We will 1) establish relative IL-12 family cytokine expression across tissue types and how obesity affects the expression, 2) determine regulation of IL-12 family cytokines in AT

during the development of obesity as these animals transition to an inflamed, insulin resistant state, and 3) elucidate the regulation and function of IL-12 family members in preadipocytes and adipocytes, as major, lineage-related cell types in AT.

Chapter III. Although IL-27 plays an important role in initiating and maintaining cell-mediated immune responses, little is known regarding IL-27 in obesity-associated inflammation in adipocytes. As our previous study demonstrated a regulatory role for IL-27 in obesity-induced inflammation, the objective of the study summarized in Chapter III is to: 1) establish divergent regulation of IL-27 genes, p28 and EBI3, in response to inflammation based on cellular phenotype, 2) determine upstream signaling pathways that regulate p28 and EBI3 expression in response to TNFα, and 3) elucidate that how HDACs are involved in epigenetic regulation of these two genes.

Chapter IV. TNFα potently regulates inflammatory gene expression and IR through interacting with TNFR1 on the surface of the plasma membrane. As receptor internalization and receptor shedding lead to dynamic turnover of receptors on the surface, vesicular trafficking plays a critical role in mediating receptor density and TNFα action. As there is evidence that the GA may play a role in TNFR trafficking as well as serving as a reservoir for TNFR1 in other cell types, the objective of the study summarized in Chapter IV is to: 1) determine a regulatory role of the GA in TNFα-induced inflammatory gene expression in adipocytes and 2) elucidate specific mechanisms involved in this regulatory process.

Table 1.1. Classification of Histone Deacetylases

Class	Enzyme	Mechanism of deacetylase activity	Subcellular localization
I	HDAC1	Zn2+ dependent	Nuclear
	HDAC2	Zn2+ dependent	Nuclear
	HDAC3	Zn2+ dependent	Nucleocytoplasmic shuttling
	HDAC8	Zn2+ dependent	Nuclear
IIa	HDAC4	Zn2+ dependent	Nucleocytoplasmic shuttling
	HDAC5	Zn2+ dependent	Nucleocytoplasmic shuttling
	HDAC7	Zn2+ dependent	Nucleocytoplasmic shuttling
	HDAC9	Zn2+ dependent	Nucleocytoplasmic shuttling
IIb	HDAC6	Zn2+ dependent	Nucleocytoplasmic shuttling
	HDAC10	Zn2+ dependent	Nucleocytoplasmic shuttling
III	SIRT1	NAD+ dependent	Nuclear
	SIRT2	NAD+ dependent	Cytosol
	SIRT3	NAD+ dependent	Mitochondria
	SIRT4	NAD+ dependent	Unknown
	SIRT5	NAD+ dependent	Unknown
	SIRT6	NAD+ dependent	Unknown
	SIRT7	NAD+ dependent	Unknown
IV	HDAC11	Zn2+ dependent	Nuclear

Table 1.2. Classification of Histone Deacetylase Inhibitors

Group	Compounds	IC50 range in vitro
Hydroxamic acids	Trichostatin A (TSA)	nM
	Suberoyl anilide bishydroxamide (SAHA)	μM
	M-carboxycinnamic acid bishydroxamide (CBHA)	μM
	Scriptaid	μM
	Pyroxamide	μM
	Oxamflatin	nM
Short-chain fatty acids	Butyrate	mM
	Phenylbutyrate	mM
	Valproric acid	mM
Cyclic tetrapeptides	Trapoxin	nM
	HC-toxin	nM
	Chlamydocin	nM
	Depudesin	μM
	Apicidine	nM-uM
	Depsipeptide	nM
Benzamides	N-acetyldinaline	μM
	MS-275	μM
Sirtuin (Class III) inhibitors	Sirtinol	
	Splitomicin	
	Nicotinamide	

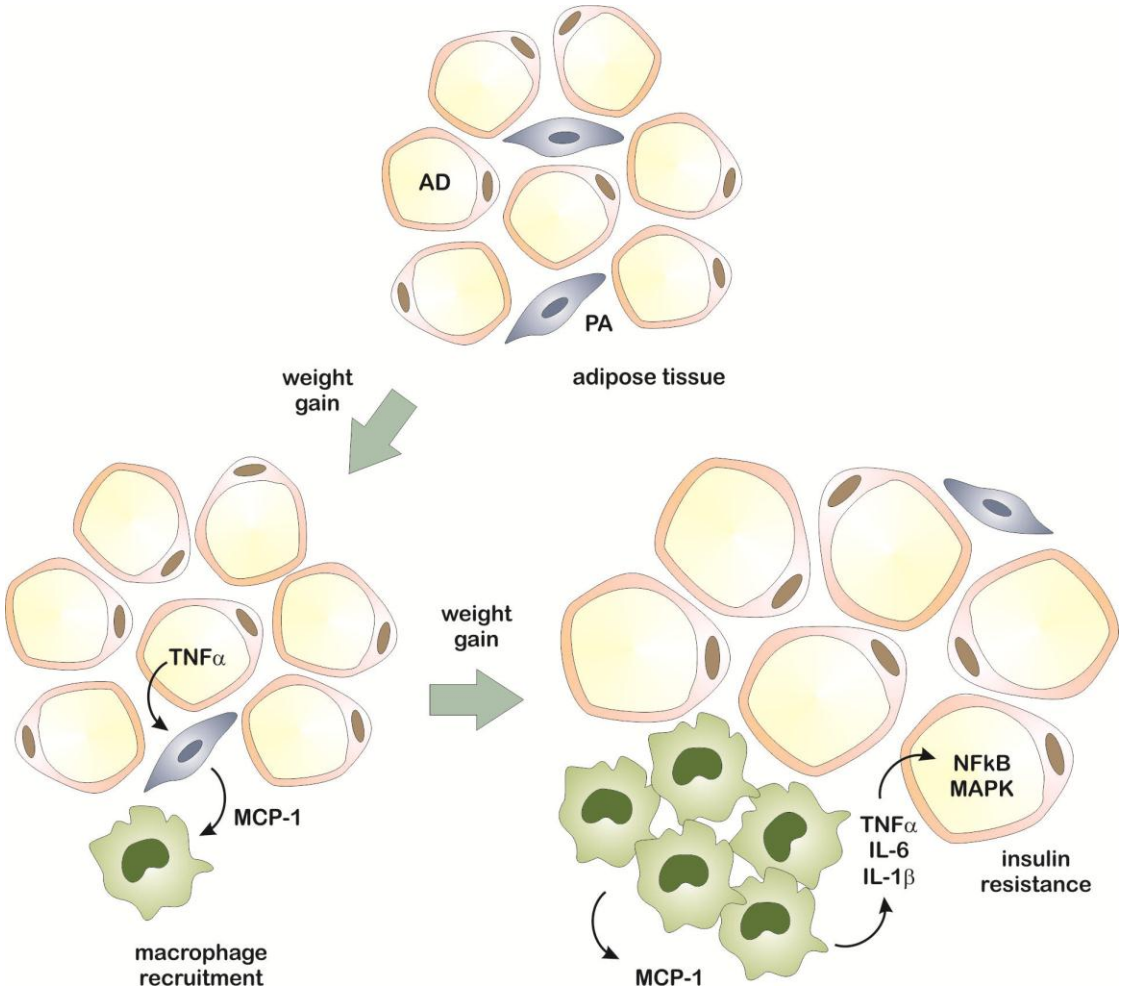

Figure 1.1. Obesity-induced adipose tissue inflammation. Obesity is characterized by expansion of AT. As adipose tissue mass is continue to increase, adipocytes can secrete chemoattractant proteins (i.e., MCP-1) to recruit macrophages into AT. Infiltrated macrophages and enlarged adipocytes secrete a large amount of TNFα that stimulates PAs and ADs to activate inflammatory signaling pathways (i.e., MAPK and NF-κB) and gene expression, contributing adipocyte dysfunction and IR.

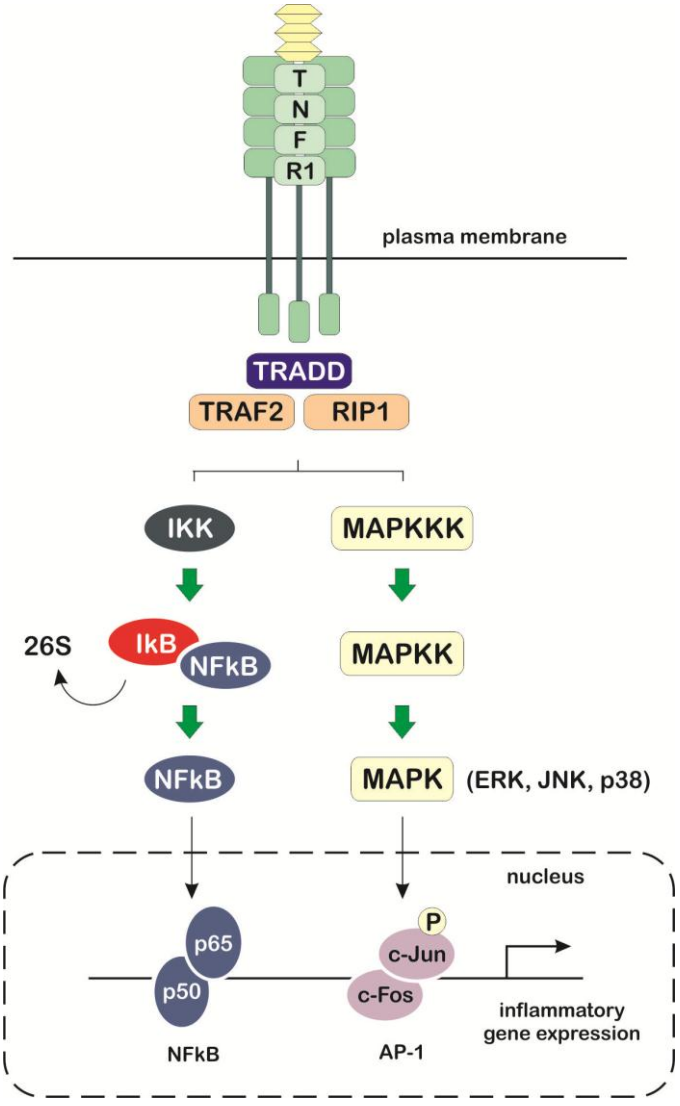

Figure 1.2. TNFα-induced inflammatory gene expression through MAPK and NF-κB signaling pathways.

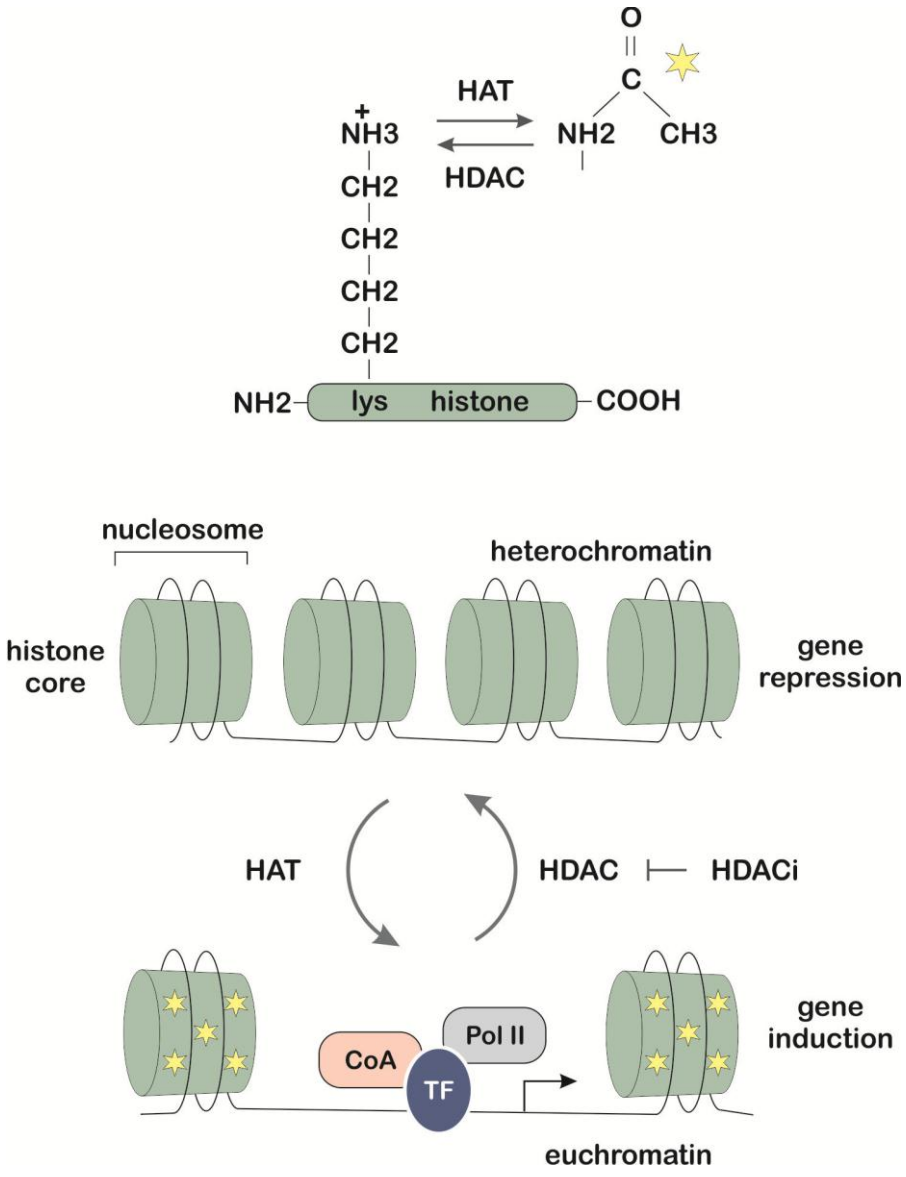

Figure 1.3. Gene activation and repression are regulated by HATs and HDACs on core histones. HATs transfer acetyl groups (yellow star) to lysine residues on proteins, and HDACs catalyze removal of these groups. HATs acetylate on lysine residues in the N-terminal tail of histone proteins. Acetylated histone proteins change the chromatin structure from the resting closed conformation to an activated open form, allowing transcription factors binding to the promoter to initiate gene transcription. HDACs convert these actions, thereby causing gene silencing. CoA: coactivator; TF: transcription factor; Pol II: RNA polymerase II.

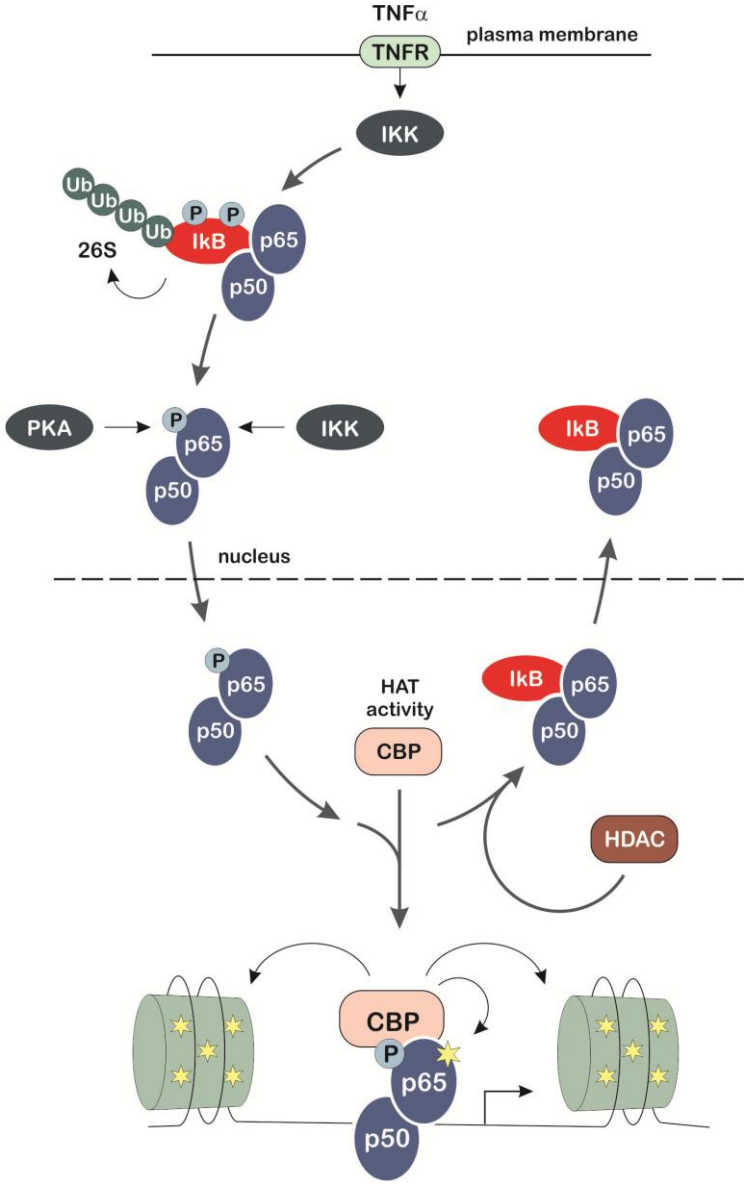

Figure 1.4. Acetylation of NF-κB for transcriptional activity. TNFα signaling activates IKK, which phosphorylates IκBα, leading to their degradation and the ensuring nuclear translocation of NF-κB and subsequent transactivation of NF-κB dependent genes. p65, the subunit of NF-κB that contains the transactivation domain, is acetylated by a general transcriptional coactivator, CBP, which possess intrinsic HAT activity. CBP also acetylates histone proteins to open up the chromatin. CBP: CREB-binding protein.

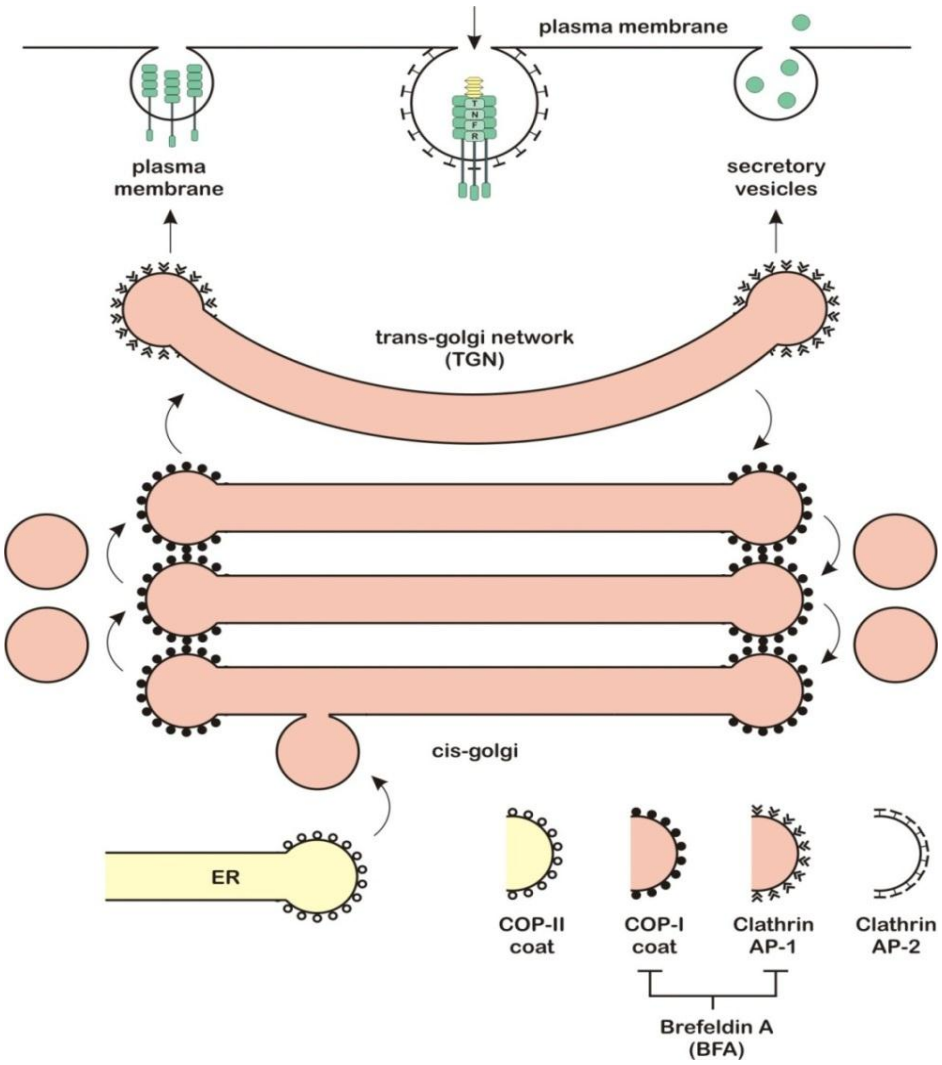

Figure 1.5. Vesicular trafficking in Golgi apparatus.

Reference List

1. Ogden CL, Carroll MD, McDowell MA, Flegal KM. Obesity among adults in the United States--no statistically significant chance since 2003-2004. NCHS Data Brief. 2007 Nov;1-8.

2. Haslam DW, James WP. Obesity. Lancet. 2005 Oct 1;366:1197-209.

3. Ogden CL, Lamb MM, Carroll MD, Flegal KM. Obesity and socioeconomic status in adults: United States, 2005-2008. NCHS Data Brief. 2010 Dec;1-8.

4. Hausman DB, DiGirolamo M, Bartness TJ, Hausman GJ, Martin RJ. The biology of white adipocyte proliferation. Obes Rev. 2001 Nov;2:239-54.

5. Villareal DT, Banks MR, Patterson BW, Polonsky KS, Klein S. Weight loss therapy improves pancreatic endocrine function in obese older adults. Obesity (Silver Spring). 2008 Jun;16:1349-54.

6. Goodpaster BH, Katsiaras A, Kelley DE. Enhanced fat oxidation through physical activity is associated with improvements in insulin sensitivity in obesity. Diabetes. 2003 Sep;52:2191-7.

7. Nath C. Literacy and diabetes self-management. Am J Nurs. 2007 Jun;107:43-9.

8. Wild S, Roglic G, Green A, Sicree R, King H. Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. Diabetes Care. 2004 May;27:1047-53.

9. Zhang P, Zhang X, Brown J, Vistisen D, Sicree R, Shaw J, Nichols G. Global healthcare expenditure on diabetes for 2010 and 2030. Diabetes Res Clin Pract. 2010 Mar;87:293-301.

10. Siiteri PK. Adipose tissue as a source of hormones. Am J Clin Nutr. 1987 Jan;45:277-82.

11. Cook KS, Min HY, Johnson D, Chaplinsky RJ, Flier JS, Hunt CR, Spiegelman BM. Adipsin: a circulating serine protease homolog secreted by adipose tissue and sciatic nerve. Science. 1987 Jul 24;237:402-5.

12. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. Nature. 1994 Dec 1;372:425-32.

13. Campfield LA, Smith FJ, Guisez Y, Devos R, Burn P. Recombinant mouse OB protein: evidence for a peripheral signal linking adiposity and central neural networks. Science. 1995 Jul 28;269:546-9.

14. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest. 2005 May;115:1111-9.

15. Schroder M, Kaufman RJ. ER stress and the unfolded protein response. Mutat Res. 2005 Jan 6;569:29-63.

16. Scheuner D, Song B, McEwen E, Liu C, Laybutt R, Gillespie P, Saunders T, Bonner-Weir S, Kaufman RJ. Translational control is required for the unfolded protein response and in vivo glucose homeostasis. Mol Cell. 2001 Jun;7:1165-76.

17. Evans JL, Goldfine ID, Maddux BA, Grodsky GM. Oxidative stress and stress-activated signaling pathways: a unifying hypothesis of type 2 diabetes. Endocr Rev. 2002 Oct;23:599-622.

18. Lamb RE, Goldstein BJ. Modulating an oxidative-inflammatory cascade: potential new treatment strategy for improving glucose metabolism, insulin resistance, and vascular function. Int J Clin Pract. 2008 Jul;62:1087-95.

19. Furukawa S, Fujita T, Shimabukuro M, Iwaki M, Yamada Y, Nakajima Y, Nakayama O, Makishima M, Matsuda M, Shimomura I. Increased oxidative stress in obesity and its impact on metabolic syndrome. J Clin Invest. 2004 Dec;114:1752-61.

20. Keaney JF, Jr., Larson MG, Vasan RS, Wilson PW, Lipinska I, Corey D, Massaro JM, Sutherland P, Vita JA, Benjamin EJ. Obesity and systemic oxidative stress: clinical correlates of oxidative stress in the Framingham Study. Arterioscler Thromb Vasc Biol. 2003 Mar 1;23:434-9.

21. Olusi SO. Obesity is an independent risk factor for plasma lipid peroxidation and depletion of erythrocyte cytoprotectic enzymes in humans. Int J Obes Relat Metab Disord. 2002 Sep;26:1159-64.

22. Liu G, Rondinone CM. JNK: bridging the insulin signaling and inflammatory pathway. Curr Opin Investig Drugs. 2005 Oct;6:979-87.

23. Shoelson SE, Lee J, Goldfine AB. Inflammation and insulin resistance. J Clin Invest. 2006 Jul;116:1793-801.

24. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003 Dec;112:1796-808.

25. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest. 2003 Dec;112:1821-30.

26. Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol. 2008 May;9:367-77.

27. Curat CA, Miranville A, Sengenes C, Diehl M, Tonus C, Busse R, Bouloumie A. From blood monocytes to adipose tissue-resident macrophages: induction of diapedesis by human mature adipocytes. Diabetes. 2004 May;53:1285-92.

28. Cancello R, Henegar C, Viguerie N, Taleb S, Poitou C, Rouault C, Coupaye M, Pelloux V, Hugol D, et al. Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. Diabetes. 2005 Aug;54:2277-86.

29. Cousin B, Munoz O, Andre M, Fontanilles AM, Dani C, Cousin JL, Laharrague P, Casteilla L, Penicaud L. A role for preadipocytes as macrophage-like cells. FASEB J. 1999 Feb;13:305-12.

30. Cousin B, Andre M, Casteilla L, Penicaud L. Altered macrophage-like functions of preadipocytes in inflammation and genetic obesity. J Cell Physiol. 2001 Mar;186:380-6.

31. Alexaki VI, Notas G, Pelekanou V, Kampa M, Valkanou M, Theodoropoulos P, Stathopoulos EN, Tsapis A, Castanas E. Adipocytes as immune cells: differential expression of TWEAK, BAFF, and APRIL and their receptors (Fn14, BAFF-R, TACI, and BCMA) at different stages of normal and pathological adipose tissue development. J Immunol. 2009 Nov 1;183:5948-56.

32. Bes-Houtmann S, Roche R, Hoareau L, Gonthier MP, Festy F, Caillens H, Gasque P, Lefebvre dC, Cesari M. Presence of functional TLR2 and TLR4 on human adipocytes. Histochem Cell Biol. 2007 Feb;127:131-7.

33. Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. Nat Rev Immunol. 2008 Dec;8:923-34.

34. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature. 1997 Oct 9;389:610-4.

35. Duffaut C, Galitzky J, Lafontan M, Bouloumie A. Unexpected trafficking of immune cells within the adipose tissue during the onset of obesity. Biochem Biophys Res Commun. 2009 Jul 10;384:482-5.

36. Duffaut C, Zakaroff-Girard A, Bourlier V, Decaunes P, Maumus M, Chiotasso P, Sengenes C, Lafontan M, Galitzky J, Bouloumie A. Interplay between human adipocytes and T lymphocytes in obesity: CCL20 as an adipochemokine and T lymphocytes as lipogenic modulators. Arterioscler Thromb Vasc Biol. 2009 Oct;29:1608-14.

37. Ohmura K, Ishimori N, Ohmura Y, Tokuhara S, Nozawa A, Horii S, Andoh Y, Fujii S, Iwabuchi K, et al. Natural killer T cells are involved in adipose tissues inflammation and glucose intolerance in diet-induced obese mice. Arterioscler Thromb Vasc Biol. 2010 Feb;30:193-9.

38. Feuerer M, Herrero L, Cipolletta D, Naaz A, Wong J, Nayer A, Lee J, Goldfine AB, Benoist C, et al. Lean, but not obese, fat is enriched for a unique population of regulatory T cells that affect metabolic parameters. Nat Med. 2009 Aug;15:930-9.

39. Nishimura S, Manabe I, Nagasaki M, Eto K, Yamashita H, Ohsugi M, Otsu M, Hara K, Ueki K, et al. CD8+ effector T cells contribute to macrophage recruitment and adipose tissue inflammation in obesity. Nat Med. 2009 Aug;15:914-20.

40. Kintscher U, Hartge M, Hess K, Foryst-Ludwig A, Clemenz M, Wabitsch M, Fischer-Posovszky P, Barth TF, Dragun D, et al. T-lymphocyte infiltration in visceral adipose tissue: a primary event in adipose tissue inflammation and the development of obesity-mediated insulin resistance. Arterioscler Thromb Vasc Biol. 2008 Jul;28:1304-10.

41. Sell H, Eckel J. Adipose tissue inflammation: novel insight into the role of macrophages and lymphocytes. Curr Opin Clin Nutr Metab Care. 2010 Jul;13:366-70.

42. Beadling C, Slifka MK. Regulation of innate and adaptive immune responses by the related cytokines IL-12, IL-23, and IL-27. Arch Immunol Ther Exp (Warsz). 2006 Jan;54:15-24.

43. Kastelein RA, Hunter CA, Cua DJ. Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation. Annu Rev Immunol. 2007;25:221-42.

44. Goriely S, Goldman M. Interleukin-12 family members and the balance between rejection and tolerance. Curr Opin Organ Transplant. 2008 Feb;13:4-9.

45. Merberg DM, Wolf SF, Clark SC. Sequence similarity between NKSF and the IL-6/G-CSF family. Immunol Today. 1992 Feb;13:77-8.

46. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol. 2003 Feb;3:133-46.

47. Langrish CL, McKenzie BS, Wilson NJ, de Waal MR, Kastelein RA, Cua DJ. IL-12 and IL-23: master regulators of innate and adaptive immunity. Immunol Rev. 2004 Dec;202:96-105.

48. Lyakh L, Trinchieri G, Provezza L, Carra G, Gerosa F. Regulation of interleukin-12/interleukin-23 production and the T-helper 17 response in humans. Immunol Rev. 2008 Dec;226:112-31.

49. Gillessen S, Carvajal D, Ling P, Podlaski FJ, Stremlo DL, Familletti PC, Gubler U, Presky DH, Stern AS, Gately MK. Mouse interleukin-12 (IL-12) p40 homodimer: a potent IL-12 antagonist. Eur J Immunol. 1995 Jan;25:200-6.

50. Heinzel FP, Hujer AM, Ahmed FN, Rerko RM. In vivo production and function of IL-12 p40 homodimers. J Immunol. 1997 May 1;158:4381-8.

51. Oppmann B, Lesley R, Blom B, Timans JC, Xu Y, Hunte B, Vega F, Yu N, Wang J, et al. Novel p19 protein engages IL-12p40 to form a cytokine, IL-23, with biological activities similar as well as distinct from IL-12. Immunity. 2000 Nov;13:715-25.

52. Pflanz S, Timans JC, Cheung J, Rosales R, Kanzler H, Gilbert J, Hibbert L, Churakova T, Travis M, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. Immunity. 2002 Jun;16:779-90.

53. Collison LW, Vignali DA. Interleukin-35: odd one out or part of the family? Immunol Rev. 2008 Dec;226:248-62.

54. Niedbala W, Wei XQ, Cai B, Hueber AJ, Leung BP, McInnes IB, Liew FY. IL-35 is a novel cytokine with therapeutic effects against collagen-induced arthritis through the expansion of regulatory T cells and suppression of Th17 cells. Eur J Immunol. 2007 Nov;37:3021-9.

55. Wirtz S, Becker C, Fantini MC, Nieuwenhuis EE, Tubbe I, Galle PR, Schild HJ, Birkenbach M, Blumberg RS, Neurath MF. EBV-induced gene 3 transcription is induced by TLR signaling in primary dendritic cells via NF-kappa B activation. J Immunol. 2005 Mar 1;174:2814-24.

56. Coulomb-L'Hermine A, Larousserie F, Pflanz S, Bardel E, Kastelein RA, Devergne O. Expression of interleukin-27 by human trophoblast cells. Placenta. 2007 Nov;28:1133-40.

57. Kempe S, Heinz P, Kokai E, Devergne O, Marx N, Wirth T. Epstein-barr virus-induced gene-3 is expressed in human atheroma plaques. Am J Pathol. 2009 Jul;175:440-7.

58. Kong W, Yen JH, Vassiliou E, Adhikary S, Toscano MG, Ganea D. Docosahexaenoic acid prevents dendritic cell maturation and in vitro and in vivo expression of the IL-12 cytokine family. Lipids Health Dis. 2010;9:12.

59. Liu J, Guan X, Ma X. Regulation of IL-27 p28 gene expression in macrophages through MyD88- and interferon-gamma-mediated pathways. J Exp Med. 2007 Jan 22;204:141-52.

60. Yoshimura T, Takeda A, Hamano S, Miyazaki Y, Kinjyo I, Ishibashi T, Yoshimura A, Yoshida H. Two-sided roles of IL-27: induction of Th1 differentiation on naive CD4+ T cells versus suppression of proinflammatory cytokine production including IL-23-induced IL-17 on activated CD4+ T cells partially through STAT3-dependent mechanism. J Immunol. 2006 Oct 15;177:5377-85.

61. Pennica D, Wood WI, Chien KR. Cardiotrophin-1: a multifunctional cytokine that signals via LIF receptor-gp 130 dependent pathways. Cytokine Growth Factor Rev. 1996 Jun;7:81-91.

62. Zvonic S, Baugh JE, Jr., Arbour-Reily P, Mynatt RL, Stephens JM. Cross-talk among gp130 cytokines in adipocytes. J Biol Chem. 2005 Oct 7;280:33856-63.

63. Winkler G, Dworak O, Salamon F, Salamon D, Speer G, Cseh K. Increased interleukin-12 plasma concentrations in both, insulin-dependent and non-insulin-dependent diabetes mellitus. Diabetologia. 1998 Apr;41:488.

64. Wegner M, Winiarska H, Bobkiewicz-Kozlowska T, Dworacka M. IL-12 serum levels in patients with type 2 diabetes treated with sulphonylureas. Cytokine. 2008 Jun;42:312-6.

65. Sumarac-Dumanovic M, Stevanovic D, Ljubic A, Jorga J, Simic M, Stamenkovic-Pejkovic D, Starcevic V, Trajkovic V, Micic D. Increased activity of interleukin-23/interleukin-17 proinflammatory axis in obese women. Int J Obes (Lond). 2009 Jan;33:151-6.

66. Wen Y, Gu J, Li SL, Reddy MA, Natarajan R, Nadler JL. Elevated glucose and diabetes promote interleukin-12 cytokine gene expression in mouse macrophages. Endocrinology. 2006 May;147:2518-25.

67. Yao K, Ge JB, Sun AJ, Hong XW, Shi HY, Huang RC, Jia QZ, Wang KQ, Zhong CP, et al. [Effects and mechanism of hyperglycemia on development and maturation and immune function of human monocyte derived dendritic cells]. Zhonghua Xin Xue Guan Bing Za Zhi. 2006 Jan;34:60-4.

68. Kim SJ, Choi Y, Choi YH, Park T. Obesity activates toll-like receptor-mediated proinflammatory signaling cascades in the adipose tissue of mice. J Nutr Biochem. 2011 Mar 15.

69. Strissel KJ, DeFuria J, Shaul ME, Bennett G, Greenberg AS, Obin MS. T-cell recruitment and Th1 polarization in adipose tissue during diet-induced obesity in C57BL/6 mice. Obesity (Silver Spring). 2010 Oct;18:1918-25.

70. Febbraio MA. gp130 receptor ligands as potential therapeutic targets for obesity. J Clin Invest. 2007 Apr;117:841-9.

71. White UA, Stewart WC, Mynatt RL, Stephens JM. Neuropoietin attenuates adipogenesis and induces insulin resistance in adipocytes. J Biol Chem. 2008 Aug 15;283:22505-12.

72. Song Y, Miyaki K, Araki J, Zhang L, Omae K, Muramatsu M. The interaction between the interleukin 6 receptor gene genotype and dietary energy intake on abdominal obesity in Japanese men. Metabolism. 2007 Jul;56:925-30.

73. Pittas AG, Joseph NA, Greenberg AS. Adipocytokines and insulin resistance. J Clin Endocrinol Metab. 2004 Feb;89:447-52.

74. Zurawska-Klis M, Drzewoski J. [Inflammatory markers and adiponectin plasma level in patients with type 2 diabetes]. Pol Arch Med Wewn. 2005 Jul;114:652-7.

75. Hotamisligil GS, Peraldi P, Budavari A, Ellis R, White MF, Spiegelman BM. IRS-1-mediated inhibition of insulin receptor tyrosine kinase activity in TNF-alpha- and obesity-induced insulin resistance. Science. 1996 Feb 2;271:665-8.

76. Gao Z, Hwang D, Bataille F, Lefevre M, York D, Quon MJ, Ye J. Serine phosphorylation of insulin receptor substrate 1 by inhibitor kappa B kinase complex. J Biol Chem. 2002 Dec 13;277:48115-21.

77. Gao N, Zhang Z, Jiang BH, Shi X. Role of PI3K/AKT/mTOR signaling in the cell cycle progression of human prostate cancer. Biochem Biophys Res Commun. 2003 Oct 31;310:1124-32.

78. Steinberg GR, Michell BJ, van Denderen BJ, Watt MJ, Carey AL, Fam BC, Andrikopoulos S, Proietto J, Gorgun CZ, et al. Tumor necrosis factor alpha-induced skeletal muscle insulin resistance involves suppression of AMP-kinase signaling. Cell Metab. 2006 Dec;4:465-74.

79. Carswell EA, Old LJ, Kassel RL, Green S, Fiore N, Williamson B. An endotoxin-induced serum factor that causes necrosis of tumors. Proc Natl Acad Sci U S A. 1975 Sep;72:3666-70.

80. Beutler B, Greenwald D, Hulmes JD, Chang M, Pan YC, Mathison J, Ulevitch R, Cerami A. Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. Nature. 1985 Aug 8;316:552-4.

81. Kawakami M, Pekala PH, Lane MD, Cerami A. Lipoprotein lipase suppression in 3T3-L1 cells by an endotoxin-induced mediator from exudate cells. Proc Natl Acad Sci U S A. 1982 Feb;79:912-6.

82. Aggarwal BB. Signalling pathways of the TNF superfamily: a double-edged sword. Nat Rev Immunol. 2003 Sep;3:745-56.

83. Baud L, Fouqueray B, Bellocq A. Cytokines and hormones with anti-inflammatory effects: new tools for therapeutic intervention. Curr Opin Nephrol Hypertens. 2001 Jan;10:49-54.

84. Sethi JK, Hotamisligil GS. The role of TNF alpha in adipocyte metabolism. Semin Cell Dev Biol. 1999 Feb;10:19-29.

85. Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM. Tumor necrosis factor alpha inhibits signaling from the insulin receptor. Proc Natl Acad Sci U S A. 1994 May 24;91:4854-8.

86. Wallach D, Varfolomeev EE, Malinin NL, Goltsev YV, Kovalenko AV, Boldin MP. Tumor necrosis factor receptor and Fas signaling mechanisms. Annu Rev Immunol. 1999;17:331-67.

87. Cawthorn WP, Sethi JK. TNF-alpha and adipocyte biology. FEBS Lett. 2008 Jan 9;582:117-31.

88. Peraldi P, Hotamisligil GS, Buurman WA, White MF, Spiegelman BM. Tumor necrosis factor (TNF)-alpha inhibits insulin signaling through stimulation of the p55 TNF receptor and activation of sphingomyelinase. J Biol Chem. 1996 May 31;271:13018-22.

89. Bradley JR. TNF-mediated inflammatory disease. J Pathol. 2008 Jan;214:149-60.

90. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993 Jan 1;259:87-91.

91. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest. 1995 May;95:2409-15.

92. Nieto-Vazquez I, Fernandez-Veledo S, Kramer DK, Vila-Bedmar R, Garcia-Guerra L, Lorenzo M. Insulin resistance associated to obesity: the link TNF-alpha. Arch Physiol Biochem. 2008 Jul;114:183-94.

93. Stephens JM, Pekala PH. Transcriptional repression of the C/EBP-alpha and GLUT4 genes in 3T3-L1 adipocytes by tumor necrosis factor-alpha. Regulations is coordinate and independent of protein synthesis. J Biol Chem. 1992 Jul 5;267:13580-4.

94. Arner P. The adipocyte in insulin resistance: key molecules and the impact of the thiazolidinediones. Trends Endocrinol Metab. 2003 Apr;14:137-45.

95. Pirola L, Bonnafous S, Johnston AM, Chaussade C, Portis F, Van OE. Phosphoinositide 3-kinase-mediated reduction of insulin receptor substrate-1/2 protein expression via different mechanisms contributes to the insulin-induced desensitization of its signaling pathways in L6 muscle cells. J Biol Chem. 2003 May 2;278:15641-51.

96. Zick Y. Insulin resistance: a phosphorylation-based uncoupling of insulin signaling. Trends Cell Biol. 2001 Nov;11:437-41.

97. Aguirre V, Werner ED, Giraud J, Lee YH, Shoelson SE, White MF. Phosphorylation of Ser307 in insulin receptor substrate-1 blocks interactions with the insulin receptor and inhibits insulin action. J Biol Chem. 2002 Jan 11;277:1531-7.

98. Zick Y. Role of Ser/Thr kinases in the uncoupling of insulin signaling. Int J Obes Relat Metab Disord. 2003 Dec;27 Suppl 3:S56-S60.

99. Paz K, Hemi R, LeRoith D, Karasik A, Elhanany E, Kanety H, Zick Y. A molecular basis for insulin resistance. Elevated serine/threonine phosphorylation of IRS-1 and IRS-2 inhibits their binding to the juxtamembrane region of the insulin receptor and impairs their ability to undergo insulin-induced tyrosine phosphorylation. J Biol Chem. 1997 Nov 21;272:29911-8.

100. Fujishiro M, Gotoh Y, Katagiri H, Sakoda H, Ogihara T, Anai M, Onishi Y, Ono H, Abe M, et al. Three mitogen-activated protein kinases inhibit insulin signaling by different mechanisms in 3T3-L1 adipocytes. Mol Endocrinol. 2003 Mar;17:487-97.

101. Stephens JM, Lee J, Pilch PF. Tumor necrosis factor-alpha-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. J Biol Chem. 1997 Jan 10;272:971-6.

102. Ruan H, Hacohen N, Golub TR, Van PL, Lodish HF. Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. Diabetes. 2002 May;51:1319-36.

103. Ruan H, Miles PD, Ladd CM, Ross K, Golub TR, Olefsky JM, Lodish HF. Profiling gene transcription in vivo reveals adipose tissue as an immediate target of tumor necrosis factor-alpha: implications for insulin resistance. Diabetes. 2002 Nov;51:3176-88.

104. Dahlman I, Forsgren M, Sjogren A, Nordstrom EA, Kaaman M, Naslund E, Attersand A, Arner P. Downregulation of electron transport chain genes in

visceral adipose tissue in type 2 diabetes independent of obesity and possibly involving tumor necrosis factor-alpha. Diabetes. 2006 Jun;55:1792-9.

105. Qatanani M, Lazar MA. Mechanisms of obesity-associated insulin resistance: many choices on the menu. Genes Dev. 2007 Jun 15;21:1443-55.

106. Coppack SW. Pro-inflammatory cytokines and adipose tissue. Proc Nutr Soc. 2001 Aug;60:349-56.

107. Hotamisligil GS. Inflammatory pathways and insulin action. Int J Obes Relat Metab Disord. 2003 Dec;27 Suppl 3:S53-S55.

108. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. A central role for JNK in obesity and insulin resistance. Nature. 2002 Nov 21;420:333-6.

109. Lee YH, Giraud J, Davis RJ, White MF. c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. J Biol Chem. 2003 Jan 31;278:2896-902.

110. Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. Nat Rev Immunol. 2008 Dec;8:923-34.

111. Pearson G, Robinson F, Beers GT, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev. 2001 Apr;22:153-83.

112. Zhang Y, Dong C. Regulatory mechanisms of mitogen-activated kinase signaling. Cell Mol Life Sci. 2007 Nov;64:2771-89.

113. Tanoue T, Nishida E. Molecular recognitions in the MAP kinase cascades. Cell Signal. 2003 May;15:455-62.

114. Bost F, Aouadi M, Caron L, Binetruy B. The role of MAPKs in adipocyte differentiation and obesity. Biochimie. 2005 Jan;87:51-6.

115. Jain RG, Phelps KD, Pekala PH. Tumor necrosis factor-alpha initiated signal transduction in 3T3-L1 adipocytes. J Cell Physiol. 1999 Apr;179:58-66.

116. Ryden M, Dicker A, van H, V, Hauner H, Brunnberg M, Perbeck L, Lonnqvist F, Arner P. Mapping of early signaling events in tumor necrosis factor-alpha - mediated lipolysis in human fat cells. J Biol Chem. 2002 Jan 11;277:1085-91.

117. Solinas G, Vilcu C, Neels JG, Bandyopadhyay GK, Luo JL, Naugler W, Grivennikov S, Wynshaw-Boris A, Scadeng M, et al. JNK1 in hematopoietically derived cells contributes to diet-induced inflammation and insulin resistance without affecting obesity. Cell Metab. 2007 Nov;6:386-97.

118. Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol. 2007 Jan;8:49-62.

119. Beg AA, Sha WC, Bronson RT, Baltimore D. Constitutive NF-kappa B activation, enhanced granulopoiesis, and neonatal lethality in I kappa B alpha-deficient mice. Genes Dev. 1995 Nov 15;9:2736-46.

120. Sha WC, Liou HC, Tuomanen EI, Baltimore D. Targeted disruption of the p50 subunit of NF-kappa B leads to multifocal defects in immune responses. Cell. 1995 Jan 27;80:321-30.

121. Weih F, Carrasco D, Durham SK, Barton DS, Rizzo CA, Ryseck RP, Lira SA, Bravo R. Multiorgan inflammation and hematopoietic abnormalities in mice with a targeted disruption of RelB, a member of the NF-kappa B/Rel family. Cell. 1995 Jan 27;80:331-40.

122. Wan F, Lenardo MJ. The nuclear signaling of NF-kappaB: current knowledge, new insights, and future perspectives. Cell Res. 2010 Jan;20:24-33.

123. Chen L, Fischle W, Verdin E, Greene WC. Duration of nuclear NF-kappaB action regulated by reversible acetylation. Science. 2001 Aug 31;293:1653-7.

124. Chen LF, Greene WC. Shaping the nuclear action of NF-kappaB. Nat Rev Mol Cell Biol. 2004 May;5:392-401.

125. Kiernan R, Bres V, Ng RW, Coudart MP, El MS, Sardet C, Jin DY, Emiliani S, Benkirane M. Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65. J Biol Chem. 2003 Jan 24;278:2758-66.

126. Gao Z, Chiao P, Zhang X, Zhang X, Lazar MA, Seto E, Young HA, Ye J. Coactivators and corepressors of NF-kappaB in IkappaB alpha gene promoter. J Biol Chem. 2005 Jun 3;280:21091-8.

127. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. Cell. 2008 Feb 8;132:344-62.

128. Ghosh S, Hayden MS. New regulators of NF-kappaB in inflammation. Nat Rev Immunol. 2008 Nov;8:837-48.

129. Smith CL, Nawaz Z, O'Malley BW. Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol. 1997 Jun;11:657-66.

130. Francis GA, Fayard E, Picard F, Auwerx J. Nuclear receptors and the control of metabolism. Annu Rev Physiol. 2003;65:261-311.

131. Christian M, Kiskinis E, Debevec D, Leonardsson G, White R, Parker MG. RIP140-targeted repression of gene expression in adipocytes. Mol Cell Biol. 2005 Nov;25:9383-91.

132. Fritah A. Control of skeletal muscle metabolic properties by the nuclear receptor corepressor RIP140. Appl Physiol Nutr Metab. 2009 Jun;34:362-7.

133. Herzog B, Hallberg M, Seth A, Woods A, White R, Parker MG. The nuclear receptor cofactor, receptor-interacting protein 140, is required for the regulation of hepatic lipid and glucose metabolism by liver X receptor. Mol Endocrinol. 2007 Nov;21:2687-97.

134. Powelka AM, Seth A, Virbasius JV, Kiskinis E, Nicoloro SM, Guilherme A, Tang X, Straubhaar J, Cherniack AD, et al. Suppression of oxidative metabolism and mitochondrial biogenesis by the transcriptional corepressor RIP140 in mouse adipocytes. J Clin Invest. 2006 Jan;116:125-36.

135. Zschiedrich I, Hardeland U, Krones-Herzig A, Berriel DM, Vegiopoulos A, Muggenburg J, Sombroek D, Hofmann TG, Zawatzky R, et al. Coactivator function of RIP140 for NFkappaB/RelA-dependent cytokine gene expression. Blood. 2008 Jul 15;112:264-76.

136. Rogatsky I, Zarember KA, Yamamoto KR. Factor recruitment and TIF2/GRIP1 corepressor activity at a collagenase-3 response element that mediates regulation by phorbol esters and hormones. EMBO J. 2001 Nov 1;20:6071-83.

137. Jepsen K, Hermanson O, Onami TM, Gleiberman AS, Lunyak V, McEvilly RJ, Kurokawa R, Kumar V, Liu F, et al. Combinatorial roles of the nuclear receptor corepressor in transcription and development. Cell. 2000 Sep 15;102:753-63.

138. Rosenfeld MG, Glass CK. Coregulator codes of transcriptional regulation by nuclear receptors. J Biol Chem. 2001 Oct 5;276:36865-8.

139. Lee SK, Kim JH, Lee YC, Cheong J, Lee JW. Silencing mediator of retinoic acid and thyroid hormone receptors, as a novel transcriptional corepressor molecule of activating protein-1, nuclear factor-kappaB, and serum response factor. J Biol Chem. 2000 Apr 28;275:12470-4.

140. Espinosa L, Ingles-Esteve J, Robert-Moreno A, Bigas A. IkappaBalpha and p65 regulate the cytoplasmic shuttling of nuclear corepressors: cross-talk between Notch and NFkappaB pathways. Mol Biol Cell. 2003 Feb;14:491-502.

141. Chen CJ, Deng Z, Kim AY, Blobel GA, Lieberman PM. Stimulation of CREB binding protein nucleosomal histone acetyltransferase activity by a class of transcriptional activators. Mol Cell Biol. 2001 Jan;21:476-87.

142. Guenther MG, Barak O, Lazar MA. The SMRT and N-CoR corepressors are activating cofactors for histone deacetylase 3. Mol Cell Biol. 2001 Sep;21:6091-101.

143. Cheng CS, Johnson TL, Hoffmann A. Epigenetic control: slow and global, nimble and local. Genes Dev. 2008 May 1;22:1110-4.

144. Mai A, Massa S, Rotili D, Cerbara I, Valente S, Pezzi R, Simeoni S, Ragno R. Histone deacetylation in epigenetics: an attractive target for anticancer therapy. Med Res Rev. 2005 May;25:261-309.

145. de Ruijter AJ, van Gennip AH, Caron HN, Kemp S, van Kuilenburg AB. Histone deacetylases (HDACs): characterization of the classical HDAC family. Biochem J. 2003 Mar 15;370:737-49.

146. Gregoretti IV, Lee YM, Goodson HV. Molecular evolution of the histone deacetylase family: functional implications of phylogenetic analysis. J Mol Biol. 2004 Apr 16;338:17-31.

147. Kurdistani SK, Grunstein M. Histone acetylation and deacetylation in yeast. Nat Rev Mol Cell Biol. 2003 Apr;4:276-84.

148. Johnstone RW. Histone-deacetylase inhibitors: novel drugs for the treatment of cancer. Nat Rev Drug Discov. 2002 Apr;1:287-99.

149. Marmorstein R. Structure of histone deacetylases: insights into substrate recognition and catalysis. Structure. 2001 Dec;9:1127-33.

150. Grozinger CM, Schreiber SL. Deacetylase enzymes: biological functions and the use of small-molecule inhibitors. Chem Biol. 2002 Jan;9:3-16.

151. Lallemand F, Courilleau D, Sabbah M, Redeuilh G, Mester J. Direct inhibition of the expression of cyclin D1 gene by sodium butyrate. Biochem Biophys Res Commun. 1996 Dec 4;229:163-9.

152. Lee SC, Magklara A, Smith CL. HDAC activity is required for efficient core promoter function at the mouse mammary tumor virus promoter. J Biomed Biotechnol. 2011;2011:416905.

153. Chen S, Bellew C, Yao X, Stefkova J, Dipp S, Saifudeen Z, Bachvarov D, El-Dahr SS. Histone deacetylase (HDAC) activity is critical for embryonic kidney gene expression, growth, and differentiation. J Biol Chem. 2011 Sep 16;286:32775-89.

154. Hubbert C, Guardiola A, Shao R, Kawaguchi Y, Ito A, Nixon A, Yoshida M, Wang XF, Yao TP. HDAC6 is a microtubule-associated deacetylase. Nature. 2002 May 23;417:455-8.

155. Fischle W, Dequiedt F, Fillion M, Hendzel MJ, Voelter W, Verdin E. Human HDAC7 histone deacetylase activity is associated with HDAC3 in vivo. J Biol Chem. 2001 Sep 21;276:35826-35.

156. Zhao X, Ito A, Kane CD, Liao TS, Bolger TA, Lemrow SM, Means AR, Yao TP. The modular nature of histone deacetylase HDAC4 confers phosphorylation-dependent intracellular trafficking. J Biol Chem. 2001 Sep 14;276:35042-8.

157. Guardiola AR, Yao TP. Molecular cloning and characterization of a novel histone deacetylase HDAC10. J Biol Chem. 2002 Feb 1;277:3350-6.

158. Kouzarides T. Acetylation: a regulatory modification to rival phosphorylation? EMBO J. 2000 Mar 15;19:1176-9.

159. Chen LF, Mu Y, Greene WC. Acetylation of RelA at discrete sites regulates distinct nuclear functions of NF-kappaB. EMBO J. 2002 Dec 2;21:6539-48.

160. Chen LF, Greene WC. Regulation of distinct biological activities of the NF-kappaB transcription factor complex by acetylation. J Mol Med (Berl). 2003 Sep;81:549-57.

161. Peng L, Seto E. Deacetylation of nonhistone proteins by HDACs and the implications in cancer. Handb Exp Pharmacol. 2011;206:39-56.

162. Glozak MA, Sengupta N, Zhang X, Seto E. Acetylation and deacetylation of non-histone proteins. Gene. 2005 Dec 19;363:15-23.

163. Yang XJ, Seto E. Lysine acetylation: codified crosstalk with other posttranslational modifications. Mol Cell. 2008 Aug 22;31:449-61.

164. Minucci S, Pelicci PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer. 2006 Jan;6:38-51.

165. Halili MA, Andrews MR, Labzin LI, Schroder K, Matthias G, Cao C, Lovelace E, Reid RC, Le GT, et al. Differential effects of selective HDAC inhibitors on macrophage inflammatory responses to the Toll-like receptor 4 agonist LPS. J Leukoc Biol. 2010 Jun;87:1103-14.

166. Quivy V, Van LC. Regulation at multiple levels of NF-kappaB-mediated transactivation by protein acetylation. Biochem Pharmacol. 2004 Sep 15;68:1221-9.

167. Liu Y, Xia X, Fondell JD, Yen PM. Thyroid hormone-regulated target genes have distinct patterns of coactivator recruitment and histone acetylation. Mol Endocrinol. 2006 Mar;20:483-90.

168. Blanchard F, Chipoy C. Histone deacetylase inhibitors: new drugs for the treatment of inflammatory diseases? Drug Discov Today. 2005 Feb 1;10:197-204.

169. Adcock IM. HDAC inhibitors as anti-inflammatory agents. Br J Pharmacol. 2007 Apr;150:829-31.

170. Calao M, Burny A, Quivy V, Dekoninck A, Van LC. A pervasive role of histone acetyltransferases and deacetylases in an NF-kappaB-signaling code. Trends Biochem Sci. 2008 Jul;33:339-49.

171. Marks PA. Discovery and development of SAHA as an anticancer agent. Oncogene. 2007 Feb 26;26:1351-6.

172. Bradner JE, West N, Grachan ML, Greenberg EF, Haggarty SJ, Warnow T, Mazitschek R. Chemical phylogenetics of histone deacetylases. Nat Chem Biol. 2010 Mar;6:238-43.

173. McKinsey TA. Isoform-selective HDAC inhibitors: closing in on translational medicine for the heart. J Mol Cell Cardiol. 2011 Oct;51:491-6.

174. Dai Y, Faller DV. Transcription Regulation by Class III Histone Deacetylases (HDACs)-Sirtuins. Transl Oncogenomics. 2008;3:53-65.

175. Bitterman KJ, Anderson RM, Cohen HY, Latorre-Esteves M, Sinclair DA. Inhibition of silencing and accelerated aging by nicotinamide, a putative negative regulator of yeast sir2 and human SIRT1. J Biol Chem. 2002 Nov 22;277:45099-107.

176. Schneider-Brachert W, Tchikov V, Neumeyer J, Jakob M, Winoto-Morbach S, Held-Feindt J, Heinrich M, Merkel O, Ehrenschwender M, et al. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. Immunity. 2004 Sep;21:415-28.

177. Schutze S, Tchikov V, Schneider-Brachert W. Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. Nat Rev Mol Cell Biol. 2008 Aug;9:655-62.

178. Chin YR, Horwitz MS. Mechanism for removal of tumor necrosis factor receptor 1 from the cell surface by the adenovirus RIDalpha/beta complex. J Virol. 2005 Nov;79:13606-17.

179. Amour A, Slocombe PM, Webster A, Butler M, Knight CG, Smith BJ, Stephens PE, Shelley C, Hutton M, et al. TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett. 1998 Sep 11;435:39-44.

180. Black RA. Tumor necrosis factor-alpha converting enzyme. Int J Biochem Cell Biol. 2002 Jan;34:1-5.

181. Jones SJ, Ledgerwood EC, Prins JB, Galbraith J, Johnson DR, Pober JS, Bradley JR. TNF recruits TRADD to the plasma membrane but not the trans-Golgi network, the principal subcellular location of TNF-R1. J Immunol. 1999 Jan 15;162:1042-8.

182. Gaeta ML, Johnson DR, Kluger MS, Pober JS. The death domain of tumor necrosis factor receptor 1 is necessary but not sufficient for Golgi retention of the receptor and mediates receptor desensitization. Lab Invest. 2000 Aug;80:1185-94.

183. Storey H, Stewart A, Vandenabeele P, Luzio JP. The p55 tumour necrosis factor receptor TNFR1 contains a trans-Golgi network localization signal in the C-terminal region of its cytoplasmic tail. Biochem J. 2002 Aug 15;366:15-22.

184. Wang J, Al-Lamki RS, Zhang H, Kirkiles-Smith N, Gaeta ML, Thiru S, Pober JS, Bradley JR. Histamine antagonizes tumor necrosis factor (TNF) signaling by stimulating TNF receptor shedding from the cell surface and Golgi storage pool. J Biol Chem. 2003 Jun 13;278:21751-60.

185. Pelham HR. Traffic through the Golgi apparatus. J Cell Biol. 2001 Dec 24;155:1099-101.

186. Barr FA. The Golgi apparatus: going round in circles? Trends Cell Biol. 2002 Mar;12:101-4.

187. Nickel W, Brugger B, Wieland FT. Vesicular transport: the core machinery of COPI recruitment and budding. J Cell Sci. 2002 Aug 15;115:3235-40.

188. Stoorvogel W, Oorschot V, Geuze HJ. A novel class of clathrin-coated vesicles budding from endosomes. J Cell Biol. 1996 Jan;132:21-33.

189. Boal F, Stephens DJ. Specific functions of BIG1 and BIG2 in endomembrane organization. PLoS One. 2010;5:e9898.

190. Radulescu AE, Siddhanta A, Shields D. A role for clathrin in reassembly of the Golgi apparatus. Mol Biol Cell. 2007 Jan;18:94-105.

191. Laclaustra M, Corella D, Ordovas JM. Metabolic syndrome pathophysiology: the role of adipose tissue. Nutr Metab Cardiovasc Dis. 2007 Feb;17:125-39.

192. Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol. 2004 Jan;25:4-7.

CHAPTER II

OBESITY-INDUCED REGULATION OF IL-12 CYTOKINE FAMILY IN INSULIN-RESPONSIVE TISSUES AND CULTURED ADIPOCYTES

Abstract

Obesity is a major public health concern worldwide that afflicts millions of individuals leading to diabetes, cardiovascular disease, hypertension, and stroke. Mounting evidence has established a role for chronic, low-grade inflammation in the development of obesity-induced insulin resistance (IR), as genetic ablation of pro-inflammatory cytokines and chemokines elevated in obesity improves insulin signaling in vitro and in vivo. Recent evidence highlights the interleukin (IL)-12 family cytokines as prospective inflammatory mediators linking obesity to IR. We examined, for the first time, the expression patterns of all IL-12 family members including receptors under conditions of obesity in the insulin responsive white adipose tissue (WAT), liver, skeletal muscle, and heart as obesity alters metabolism of these tissues leading to IR and diabetes. We demonstrate that IL-12 family cytokines and their cognate receptors were all expressed in the insulin responsive tissues examined, where eight of ten genes are most abundantly expressed in WAT. Moreover, we show that IL-12 family cytokines and receptors were divergently regulated under conditions of obesity in a tissue-specific manner. As most of the genes analyzed were predominantly expressed in WAT, we demonstrate that one family member, IL-27 was highly regulated in WAT based on the developmental stage of obesity as well as the inflammatory progression associated with

obesity. We further report that IL-27 is secreted from adipocytes in response to inflammatory stress and differentially regulates pro-inflammatory gene expression based on environment. Collectively, these findings suggest that divergent regulation of IL-12 family cytokines, particularly IL-27 may contribute to tissue-specific inflammation during obesity.

Introduction

Obesity and diabetes mellitus are major public health concerns worldwide leading to neuropathies, cardiovascular disease, hypertension, and stroke (1,2). Mounting evidence has established a role for chronic, low-grade inflammation in the development of obesity-induced IR (3-5). Several investigations have shown that chemokine and cytokine levels are increased during obesity, while ablation of any of these pro-inflammatory molecules improves insulin signaling in insulin responsive tissues (6,7). While WAT is recognized as a key site of cytokine expression in obesity, other insulin responsive tissues, such as liver, muscle, and heart also experience increased inflammation in the obese state (3,8-12). Therefore, obesity induces an insulin-resistant state in these tissues, resulting in an imbalance of systemic glucose homeostasis, consequently contributing to the pathological metabolic disorders associated with obesity. While it is now well recognized that an array of inflammatory cytokines and chemokines are increased in obese tissues, extensive studies are underway to identify inflammatory mediators that contribute to inflammation and ultimately chronic diseases like type 2 diabetes.

Recent evidence highlights the IL-12 family cytokines as prospective regulators linking obesity to IR. While plasma levels of select IL-12 family members are elevated

with obesity, diabetes, and metabolic syndrome (13-16), their cellular origin has not been fully determined. IL-12 family cytokines are mostly expressed in antigen-presenting cells and play critical roles during inflammatory stress. The members of the IL-12 family are heterodimeric proteins consisting of an alpha chain and a beta chain (Fig.2.1). The alpha chains consist of p19, p28, or p35 that share structural homology with IL-6, whereas the beta chains, p40 or Epstein-Barr virus-induced gene 3 (EBI3), shares homology with soluble cytokine receptor chains, such as IL-6Rα (17,18). Dimerization of specific alpha chain and beta chain subunits form the IL-12 family that includes IL-12 (i.e., p35 and p40), IL-23 (i.e., p19 and p40), IL-27 (i.e., p28 and EBI3), and the newly identified IL-35 (i.e., p35 and EBI3). Each cytokine signals through unique heterodimeric receptors composed of IL-12Rβ1, IL-12Rβ2, IL-23R, gp130, and WSX-1 (19). Evidence demonstrates that alpha and beta subunits must dimerize within the cell to produce and secrete biologically active cytokines known to play roles in linking innate resistance and adaptive immunity (20). Although the pairing between cytokine subunits and receptors is conserved, the origin, activity, and kinetic expression are cell-type and condition-specific.

IL-27 is a unique IL-12 family member, as reports have discerned two distinct functions in its regulation of the immune response: one as an initiator and the other as an attenuator of inflammatory stress (21-23). IL-27, composed of p28 and EBI3, initiates its actions through cell-surface binding to its receptor composed of WSX-1 and gp130, where gp130 is required for both ligand binding and signal transduction. While historic literature demonstrate that IL-27 expression is restricted to myeloid cells including monocytes, monocyte-derived dendritic cells, and macrophages induced mainly by toll-like receptor stimulation (21), more recent studies indicate regulation of IL-27 in other cell types. For example, p28 and EBI3 were highly expressed in regions of vascular

smooth muscle cells and endothelial cells with pro-inflammatory stimuli (i.e., TNFα and IFNγ) (24). Activation of the NF-κB signaling pathway by various inflammatory stimuli largely account for the transcriptional regulation of IL-27 (24,25). Plasma levels of several IL-12 family cytokines have been shown to be elevated in obesity and diabetes, yet their cellular origin has not been determined (13,14,26). As adipocytes displays macrophage-like features (27), expressing many of the same inflammatory genes, it could be hypothesized that several IL-12 family cytokines are expressed and secreted from adipocyte lineage and involved in obesity-associated inflammatory process. Additionally, evidence has established a mechanistic role for the gp130 cytokine in obesity-induced IR (28-30). These findings support a role for IL-27, in response to inflammatory stress, as an important mediator linking excess nutrient intake to IR and diabetes.

In this investigation, we examined the regulation of the IL-12 family cytokines and their cognate receptors in response to obesity in WAT, liver, muscle, and heart, as these insulin responsive tissues centrally regulate glucose homeostasis and vascular function (11,31,32). As we report that IL-12 family expression is most abundant in WAT, this report further investigated the developmental role of obesity on IL-12 family regulation in WAT. With a potential role for IL-12 family cytokines in WAT, we used 3T3-L1 adipocytes to show the regulatory role for IL-12 family, with focus on IL-27 in response to inflammation. This report supports a role for IL-12 family members, particularly IL-27, as important mediators linking obesity to inflammatory diseases.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DMEM), calf bovine serum (CS), Trypsin-EDTA, and recombinant murine TNFα were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from HyClone. The following antibodies were used for immunoblot analysis: Phospho-STAT1 (Tyr701), phospho-STAT3 (Tyr705), phospho-ERK (Thr202/Tyr204), phosphor-p38 (Thr180/Tyr182), phosphor-JNK (Thr183/Tyr185), and IκBα (Cell Signaling). Recombinant murine IL-27 was obtained from R&D Systems and lipopolysaccharide (LPS) was from Sigma. Enhanced chemiluminescence (ECL) reagents were obtained from Perkin-Elmer Life Sciences. All TaqMan primer probes used in this study were purchased from Applied Biosystems.

Mice and experimental diets. Animals used for this study include genetically obese male B6.V-Lepob/J (B6-ob/ob) mice and their lean littermates as well as C57BL/6J mice rendered obese by diet and their lean controls. All mice were housed and treated by the supplier (Jackson Laboratories, Bar Harbor, Maine) until shipment 1 wk prior to tissue harvest. B6-ob/ob mice and lean littermates were purchased for experimentation at 6 wks and 10 wks of age and given free access to a standard laboratory chow diet. C57BL/6J mice subjected to diet-induced obesity (DIO) were fed a high fat diet (HFD) consisting of 60% kcal from fat (Research Diets Inc. D12492) from 6 wks of age. Lean C57BL/6J control mice were fed a control low fat diet (LFD) consisting of 10% kcal from fat (Research Diet Inc. D12450B) from 6 wks of age. Both diets contained 10% kcal from protein with the balance in caloric value provided by differences in carbohydrate content. Mice receiving both diets were given free access to food and shipped for experimentation at 18 wks and 24 wks of age. All animals were

euthanized by CO_2 gas asphyxiation and epididymal AT, liver, calf skeletal muscle, and ventricular heart tissue collected and processed for preparation of total RNA. Animal care and use was in compliance with the Institute of Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Use and Care Committee.

Cell culture. The murine 3T3-L1 cell line was purchased from Howard Green, Harvard Medical School (33). Cells were propagated in DMEM supplemented with 10% CS until reaching density-induced arrest, as previously described (34). At 2 days post-confluence, growth medium was replaced with DMEM supplemented with 10% FBS, 0.5 mM 1-methy-3-isobutylxanthane, 1 µM dexamethasone, and 1.7 µM insulin (MDI) for 2 days. Subsequently, cells were cultured in DMEM supplemented only with 10% FBS over the following six days as PAs differentiated into mature ADs. Throughout the study, 'time 0' refers to density arrested cells immediately before the addition of MDI to the culture medium. Experiments described herein were conducted in density-arrested day 0 (d0) PAs or day 8 (d8) ADs. All experiments were repeated 2-3 times to validate results and ensure reliability.

Immunoblotting. Cell monolayer were washed with phosphate-buffer saline (PBS) and scraped into ice-cold lysis buffer containing 0.1 M Tris (pH 7.4), 150 mM NaCl, 10% sodium dodecyl sulfate (SDS), 1% Triton X, 0.5% Nonidet P-40 (NP40), 1 mM EDTA, 1 mM EGTA. Phosphatase inhibitors (20 mM β-glycerophosphate, 10 mM sodium fluoride and 2 µM sodium orthovanadate) and protease inhibitors (0.3 µM aprotinin, 21 µM leupetin, 1 µM pepstatin, 50 µM phenanthroline, 0.5 µM phenylmethylsulfonyl fluoride) were added to lysis buffer immediately prior to cell harvest. Cell lysates were sonicated

and centrifuged (13,000*g,* 10 min, 4ºC), and the supernatant transferred to a fresh tube. Protein content was determined by bicinchoninic acid (BCA) procedures according to manufacturer's (Pierce, Rockford, IL) instructions. Equal amounts of whole cell lysate protein were separated by SDS-PAGE electrophoresis. Cell lysates were mixed with loading buffer containing 0.25M Tris (pH6.8), 4% SDS, 10% glycerol, 0.01% bromophenol blue, and 10% dithiothreitol, then heated at 80ºC for 5 min prior to electrophoresis. Proteins were resolved on SDS-polyacylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore corp., Billerica, MA). After transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4ºC. Membranes were subsequently probed with horseradish peroxidase-conjugated secondary antibodies for 1hr at room temperature. Membranes were immersed in ECL and data visualized by autoradiography using CL-XPosure film (Pierce).

Real-Time RT-PCR. For animal study, total RNA was isolated from epididymal AT, liver, skeletal muscle, and heart by utilizing Trizol reagent according to manufacturer's protocol, and processed as described by Qiagen RNA clean-up protocol. For cell study, total RNA was extracted and genomic DNA contamination was removed using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), according to manufacturer protocol. Total RNA quality was assessed via RNA integrity gels and total RNA was quantified with a Nanodrop ND-1000 spectrophotometer. Total RNA was reverse-transcribed to cDNA in a 10 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems). The reverse transcription (RT) master mix containing RT buffer, deoxyribonucleotide triphosphate (dNTP) mix, RT random primers, RNase inhibitor (1.0 U/µl), and MultiScribe RT was added to 1 µg RNA and RNase-free

water. Reverse transcription reaction conditions followed the protocol (25℃ for 10 min, 37℃ for 120 min, 85℃ for 5 sec, followed by 4℃ in definitely/ RT complete) and utilized the Gene Amp PCR System 9700 thermal cycler (Applied Biosystems) for cDNA synthesis.

PCR amplification was run utilizing the 7500 fast system (Applied Biosystems) that consisted of enzyme activation at 95℃ for 20 sec, followed by 40 cycles of denaturation at 95℃ for 3 sec combined with annealing /extension at 60℃ for 30 sec. All data were analyzed with the ABI 7500 real time PCR system. All TaqMan primer probes used in this study were also purchased from ABI. Data were recorded and analyzed with ABI Sequence Detector Software and graphs visualized with SigmaPlot software. All data were presented as mean ± standard error of the mean (SEM) and representative of duplicate determinations. Data were normalized to 18S and measured as relative differences using the $2^{-\Delta\Delta C_T}$ method as previously described (35,36).

Statistical analyses were conducted using SPSS v18. Differences in gene expression between lean and obese animals as well as between PAs and ADs were determined via student's *t*-test where a *p*-value of <0.05 was considered significant. Other data were analyzed using analysis of variance (ANOVA), with Tukey's *post-hoc* analysis used when the *p*-value for the respective parameter was statistically significant ($p < 0.05$).

ELISA. PAs and ADs were treated with or without TNFα (100 pM) and LPS (100 ng/ml) for 12, 24, and 36 hrs. Supernatants were collected and subjected to sandwich ELISA. Cytokine (IL-12p70 and IL-27p28) levels in tissue culture media were determined by sandwich ELISA as described by the manufacturer (R & D systems). Cytokine IL-23 (p19/p40) levels in tissue culture media were determined by sandwich ELISA as

described by the manufacturer (Biolegend). Optical densities were determined using a PowerWave microplate spectrophotometer (BioTek) at 450 nm. For the wavelength correction, 570 nm was used for correction for optical imperfections in the plate. Data were analyzed using a four parameter logistic (4-PL) curve-fit. Cytokine concentrations in media were determined from standards containing known concentrations of the proteins.

Results

Relative IL-12 family cytokine and receptor gene expression across insulin responsive tissues from lean C57BL/6J mice. As recent evidence has demonstrated a potential role for IL-12 family cytokines in obesity-induced IR (13,14), we began these studies by determining the gene expression profile across four major insulin responsive tissues including white adipose tissue (WAT), liver, skeletal muscle and heart isolated from 10 wk old lean C57BL/6J mice where cytokine and receptor mRNA abundance was normalized to 18S rRNA and expressed as fold-differences relative to WAT. As illustrated in Fig.2.2, all cytokine and receptor subunits were expressed most abundantly in WAT with the exception of p35 that was ~90-fold more abundant in skeletal muscle and IL-12Rβ1 that was ~3-fold more abundant in liver. Surprisingly similar patterns of tissue distribution were noted for several genes including EBI3, p40, p19, WSX-1 where relative mRNA was most abundant in WAT and least abundant in skeletal muscle as well as IL-12Rβ2 and IL-23R that were markedly more abundant in WAT relative to all other tissues examined.

Relative IL-12 family cytokine and receptor gene expression within insulin-responsive tissues comparing lean and obese Ob/Ob mice. While IL-12 family cytokines are well established in critical roles during inflammatory stress involving antigen-presenting and other immune cells, the role of these cytokines regarding obesity-induced chronic, low-grade inflammation in metabolically active tissues remains poorly understood. Therefore, we next examined the impact of obesity on the expression of IL-12 family cytokines and receptors in each of the insulin-responsive tissues discussed above. For this study, we compared 10 wk old lean, wildtype mice and obese, leptin-deficient (ob/ob) mice given ad libitum access to standard chow. Using this genetic model of obesity, we determined relative mRNA abundance for each cytokine and receptor where obese values were expressed as fold-differences relative to lean within each tissue. As shown in Fig.2.3, WAT and skeletal muscle presented with similar profiles of gene expression where p28, EBI3 and p40 were significantly induced in obese relative to lean tissue. These data were particularly interesting as these two tissues are well established as critical mediators of obesity-induced inflammation. This is in striking contrast to heart tissue where the same three cytokine subunits and their cognate receptors were significantly suppressed with obesity. Although well established as an insulin-responsive tissue, there are no known reports demonstrating a role heart tissue in obesity-induced inflammation. As an important target of inflammatory cytokine action regarding insulin-sensitivity, it was also interesting to note that skeletal muscle presented with the greatest number of receptor subunits induced with obesity.

Relative IL-12 family cytokine gene expression in WAT during the progressive development of genetic and diet-induced obesity. It is well recognized that WAT is a key site for obesity-induced inflammation leading to systemic IR (11,32).

As data presented above demonstrated that IL-12 family cytokines were abundantly expressed in WAT and induced with genetic obesity, we next examined the progression of obesity on IL-12 family cytokine expression in WAT using two distinct stages and models of obesity (genetic vs. diet-induced obesity). According to the supplier, ob/ob mice exhibit hyperplasia with ensuing obesity notable at 1 month of age and transient glucose intolerance that begins at ~6 wks of age and subsides between 12-16 wks of age. Thus, for comparison of progressive development, experiments were conducted with 6 wk and 10 wk old ob/ob mice and wildtype littermates, representing sequential, progressive stages of obesity with developing obesity-related metabolic disorders. For development of diet-induced obesity (DIO), C57BL/6J male mice were fed a high fat diet (HFD; 60% kcal from fat) by the supplier starting at 6 wks of age. Control littermates were fed a control diet containing 10% kcal from fat. Studies were conducted at 18 wks and 24 wks of age, representing 12 wks and 18 wks of dietary intervention, respectively. Progressive stages of obesity development in both models were arbitrarily designated as stage I and stage II. Using these models, we determined relative mRNA abundance for each IL-12 family cytokine as well as other well-characterized inflammatory cytokines in WAT where fold-differences were determined between lean and obese values at each stage of obesity development. Adipsin was included as a biochemical marker of obesity as others have documented a marked suppression of this gene in WAT of obese versus lean animals (37,38).

As illustrated in Fig.2.4, mRNA expression of all IL-12 family cytokines showed no marked difference with stage I genetic (ob/ob) obesity. This was consistent with other inflammatory genes where TNFα and IL-6 were also not significantly different between lean and ob/ob mice at this stage of development. In contrast, however, relative mRNA

abundance of p28, EBI3, and p40 was significantly induced in stage II ob/ob mice, paralleling the induction of TNFα and IL-6 mRNA expression. Although not apparent as illustrated, adipsin mRNA abundance progressively decreased as ob/ob mice transitioned from stage I (40-fold reduction) to stage II (80-fold reduction) obesity. Regarding DIO, EBI3 and p40 were significantly induced after 12 wks of HFD (stage I), concomitant with induction of IL-6 and TNFα. Similar to stage II genetic obesity, relative mRNA abundance of p28, EBI3, and p40 was significantly elevated after 18 wks of HFD (stage II). Consistent with the progressive increase in IL-12 family expression, TNFα expression progressively increased (3-fold vs. 6-fold) and adipsin expression progressively decreased (7-fold vs. 25-fold) with progressive DIO. Collectively, these data demonstrated that gene expression of several IL-12 family cytokine subunits are induced at the level of gene expression in WAT based on the progressive stage of obesity development as well as the progressive inflammatory environment associated with sequential stages of obesity development.

Relative IL-12 family cytokine and receptor gene expression in 3T3-L1 adipocytes comparing undifferentiated preadipocyte (PA) and mature adipocyte (AD) mRNA abundance. The progression of obesity involves enlargement of adipose tissue mass through an increase in size and number of adipocytes as well as recruitment of macrophages that contribute to systemic circulating inflammatory cytokines originating from adipose tissue. To investigate a potential role for adipocytes in IL-12 family gene expression as observed above for WAT, we next examined relative mRNA abundance for each cytokine and receptor in 3T3-L1 murine adipocytes where values observed in mature, differentiated adipocytes (AD) were expressed as fold-differences relative to fibroblast-like, undifferentiated preadipocytes (PA). Both cell populations were rendered

quiescent either by density arrest as was the case for PAs or terminal growth arrest following 8 days of differentiation in ADs minimizing any influence of cell proliferation gene expression. As shown in Fig.2.5, differentiation had dramatic and opposite effects on the relative mRNA abundance of p28 and EBI3, where p28 mRNA was markedly more abundant and EBI3 markedly less abundant in ADs compared to PAs. Interestingly, p28 was the only gene of the IL-12 family cytokine and receptor subunits examined that increased with differentiation. More to this point, differentiation actually presented with a decrease in mRNA of WSX-1 and gp130 as well as IL-12Rβ1 and IL-23R, the receptor subunits of IL-27 and IL-23, respectively, suggesting the possibility that PAs may be more susceptible than ADs to IL-12 family cytokines stimulation.

Relative IL-12 family cytokine gene expression in PAs and ADs under conditions of inflammatory stress. As we observed a greater impact of obesity progression on IL-12 cytokine relative to receptor gene expression in WAT (Fig.2.3), we next determined the impact of inflammatory stress on relative IL-12 cytokine mRNA abundance in PAs and ADs as described above. For these determinations, total RNA was initially harvested from cells over time following stimulated with either 100 pM TNFα or 100 ng/ml LPS to determine the time of peak mRNA induction (not shown). This was followed by harvesting total RNA at times of peak induction following stimulation with TNFα or LPS or the combination of these two potent inflammatory mediators. Relative mRNA abundance was determined for each cytokine where the impact of each agonist was expressed as fold-differences relative to untreated (UT) controls. As illustrated in Fig.2.6, stimulation with TNFα or LPS markedly increased the mRNA abundance of all five IL-12 family cytokine subunits in a surprisingly similar pattern between PAs and ADs with one exception. While p28 mRNA increased nearly 500-fold when stimulated with

both TNFα and LPS in PAs, the same inflammatory agonists elicited only a 5-fold increase in mRNA in ADs. This observation is particularly interesting as we demonstrated above that p28 mRNA was elevated in ADs compared to PAs suggesting that this cytokine subunit becomes refractory to inflammatory stimuli with differentiation. These determinations also demonstrated a markedly synergistic effect of both inflammatory mediators in combination with the exception of EBI3 and p35 where TNFα stimulation alone was nearly equivalent to stimulation with both TNFα and LPS.

Accumulation of IL-12 family cytokines in culture media of PAs and ADs under conditions of inflammatory stress. As inflammatory stimuli elevated the mRNA abundance of all five IL-12 family cytokine subunit genes, we next assessed the impact of inflammatory stress on the protein accumulation of IL-12, IL-23, and IL-27 in the culture media by ELISA. As functionality of these cytokines depends on heterodimeric partnering of specific alpha and beta chain subunits, ELISAs measuring both proteins were used for IL-12 and IL-23. As this co-detection technique was not commercially available for IL-27, ELISA measuring only p28 protein was utilized instead. As shown in Fig.2.7, IL-27 as measured by p28 protein detection accumulated in the culture media in a time-dependent manner following stimulation with TNFα and LPS. As others have demonstrated that secretion is dependent on prior dimer formation, these data likely represented secretion of functional IL-27 protein comprised of p28 and EBI3 heterodimers. Neither IL-12 nor IL-23 was detected in the culture media over a 36 hr period following inflammatory insult.

Effect of IL-27 stimulation on inflammatory signaling pathways. As we demonstrated that IL-27 receptor subunit mRNA was more abundant in PAs than ADs

(Fig.2.5), we next determine the effect of IL-27 stimulation on signaling pathways that are known to be regulated by this cytokine. For these determinations, cell lysates were collected over time from PAs stimulated with 50 nM IL-27 and immunoblotted for phosphorylated and activated forms of STAT and MAPK proteins as well as the degradation of IκBα as an indirect indicator of NF-κB signaling. As illustrated in Fig.2.8, IL-27 stimulation resulted in transient accumulation of p-ERK, p-JNK, and p-STAT3 with peak accumulation at 15, 30, and 60 mins, respectively. Phosphorylation of STAT1 and p38 was not detected and a minimal decrease in IκBα from IL-27 stimulation was observed.

Effect of IL-27 stimulation on basal inflammatory gene expression. As we demonstrated above that IL-27 was capable of eliciting signaling pathways that link cytokine action to inflammatory gene expression, we next examine the effect of IL-27 stimulation on basal inflammatory gene expression. Total RNA was extracted from cells over time following stimulation with 50 nM IL-27. Relative mRNA abundance at each successive time point was expressed as fold-differences relative to levels observed prior to stimulation (0 hr). As shown in Fig.2.9, IL-27 stimulation resulted in a decrease in mRNA abundance for each of four classic inflammatory genes of MCP-1, IL-6, COX-2, and TNFα. In each case, the effect was maximal within 3 hrs and was sustained throughout the 24hr period under study suggesting a potential anti-inflammatory role of IL-27 under these conditions.

Effect of IL-27 stimulation on cytokine-mediated inflammatory gene expression. We next determined the effect of IL-27 stimulation on cytokine-mediate inflammatory gene expression with the same four genes examined above. Total RNA

was extracted from PAs at 2 hrs post-stimulation with 100 pM TNFα or 50 nM IL-27 or in combination. Relative mRNA abundance was determined for each cytokine where the impact of each agonist was expressed as fold-differences relative to untreated (UT) controls. As illustrated in Fig.2.10, TNFα elicited a marked increase in mRNA for all four inflammatory genes previously determined to peak at 2 hrs post-stimulation. While IL-27 alone had no effect on gene expression, it markedly increased the effect of TNFα on mRNA accumulation suggesting a potential pro-inflammatory role of IL-27 under these conditions.

Discussion

In this study, we present the first empirical evidence that IL-12 family cytokines and their cognate receptors are divergently regulated in insulin responsive tissues under conditions of obesity. First, we report that all IL-12 family cytokines and cognate receptors are expressed well within detectable ranges in the insulin responsive tissues with most of this family of cytokines abundantly expressed in WAT. Second, we show that IL-27 is greatly regulated in WAT dependent on the developmental stage of obesity and the inflammatory progression associated with obesity. Third, our data demonstrate that among others, IL-27 is highly regulated and secreted from cultured adipocytes and plays an important role in regulating inflammation in adipocytes. Collectively, these data provide insight to possible roles of IL-12 cytokine family, particularly IL-27, in AT under conditions linking obesity with inflammatory diseases.

Data presented in this study demonstrate the impact of obesity on the expression of IL-12 family cytokines in major insulin responsive tissues that play a critical role in

regulating metabolic homeostasis. Emerging studies highlight a role for select IL-12 family cytokines in obesity-related diseases of several key insulin responsive tissues, supporting the tissue-specific roles of IL-12 family cytokines in mediating obesity-related inflammatory diseases and metabolic dysfunctions. For example, Li et al. have shown that up-regulation of IL-12 expression in liver of obese animals leads to alterations in hepatic immune system that consequently contribute to obesity-associated liver disease (39,40). Moreover, under conditions of obesity, levels of IL-12 have been shown to be associated with IR and increased AT inflammation (14-16,41,42). Data presented here also provide novel evidence of regulation of both cytokine and receptor by obesity in a tissue-specific manner. We show that elevated expression of IL-27 (i.e., p28 and EBI3) and IL-23 (i.e., p40 and p19) as well as their receptors (i.e., WSX-1, gp130, IL-12Rβ1, and IL-23R) in skeletal muscle with genetic obesity suggests a possible autocrine and/or paracrine role for these cytokines in skeletal muscle under conditions of obesity. Of the four tissues examined in this study, the heart was the only tissue that showed suppression of IL-27 genes with obesity. It has been reported that IL-27 has dual functions involved in pro- and anti-inflammatory processes (21,22,43). While the mechanisms underlying its anti-inflammatory properties are not yet clear, it has been observed that IL-27 suppresses several key inflammatory cytokines, such as TNFα, IL-6, and IL-17 (44-47). Suppression of IL-27 in heart with obesity, therefore, potentially exacerbates the inflammatory response by inhibiting IL-27 suppression of TNFα and IL-6, two major players in vascular endothelial inflammation and atherosclerosis (48,49). Collectively, these data suggest a potential role of select IL-12 family cytokines in tissue-specific regulation with obesity.

Recent studies demonstrate that one subunit of IL-27, p28 acts as a natural antagonist of gp130-mediated signaling (50), while others show that p28 and EBI3 are expressed independently in some cell types (51) and differentially regulated in response to various stimuli (52). Data presented here show that p35 was highly expressed in skeletal muscle, while either of its partners, (i.e., p40 or EBI3), were minimally expressed in the same tissue. However, p40 and EBI3 were induced with obesity in skeletal muscle while p35 expression was not affected by the same conditions. These data suggest that the p35 chain may have a function within this tissue independent of its regulatory capacity through IL-12 or IL-35. However, under conditions of obesity, p35 may bind with inducible p40 or EBI3 to form IL-12 or IL-35, respectively in skeletal muscle. These observations suggest that the individual subunits of IL-12 family cytokines play distinct functions in tissue-specific and condition-specific manners. While roles for individual IL-12 family cytokine subunits have been studied in vitro, our in vivo data support observations that highlight the biological complexity regarding this cytokine family and remain to define their additional biological activities involved in metabolic inflammatory diseases.

Chronic inflammation is an important element of pathogenic mechanisms linking obesity and metabolic diseases such as IR and type 2 diabetes. While various metabolically critical tissues are involved during the development of metabolic diseases, WAT is recognized as the predominant site for the secretion of inflammatory mediators that cause IR and systemic metabolic dysfunction (32,53). Moreover, numerous studies have shown that WAT inflammation is progressively developed during the course of obesity and the degree of obesity is positively associated with inflammatory state in WAT as macrophage infiltration into WAT gradually increases with the development of obesity

(54-57). To address correlations between WAT inflammation and the degree of obesity, we utilized expression patterns of three key inflammatory cytokines as well as adipsin as a biochemical maker for indicating degrees of obesity in WAT of two sequential stages (i.e., stage I and stage II) of genetic and diet-induced obese mice. Data from these studies demonstrate that MCP-1 was the earliest and only inflammatory maker elevated in stage I B6-ob/ob mice (6 wk old) while all three inflammatory markers (i.e., MCP-1, IL-6, and TNFα) progressively increased when these mice reached stage II (10 wk old). These data also support those of other reports showing that MCP-1 expression is increased during initial stages of obesity to attract macrophages into WAT exacerbating the inflammatory process (53,56,58). Although mice fed a HFD show increased MCP-1, IL-6, and TNFα at both stages of development, the expression levels are progressively increased with sequential stages. These data collectively demonstrate that development of obesity is positively correlated with WAT inflammation that develops progressively over time.

Data presented here demonstrate that several IL-12 family cytokines were abundantly expressed and divergently regulated in WAT based on stage of obesity and obesity-associated inflammation. Of the four cytokines, we show that IL-27 is significantly induced in WAT of stage II genetic- and diet-induced obese mice, paralleling the induction of key pro-inflammatory cytokines (i.e., TNFα, IL-6, and MCP-1). These findings suggest that IL-27 is involved in an advanced stage of obesity-induced inflammation in WAT that can modulate insulin sensitivity and glucose metabolism of this tissue. We also show up-regulation of p40, but not p35 expression in WAT with obesity where inflammatory genes IL-6, TNFα, and MCP-1 were elevated. Consistent with our findings, a recent study showed that DIO and ob/ob mice exhibited significantly

increased mRNA levels of p40 as well as IL-6 and TNFα in epididymal WAT, but no change was observed in p35 expression (41,59). We further report that expression of EBI3 and p40 as well as pro-inflammatory genes were elevated in stage I DIO, not ob/ob mice. These data suggest that HFD, containing high amount of saturated fat, triggers inflammation leading to upregulation of these genes in WAT as others have reported that high-fat feeding increased toll-like receptor signaling leading to inflammation in WAT (41,60). In contrast, p28 expression was only upregulated in stage II obesity in both animal model systems, suggesting that advanced stages of obesity may be responsible for elevated p28 gene expression in WAT.

As we demonstrate that of the four cytokines, IL-27 is highly induced in AT with obesity, we show unique regulation of this cytokine in cultured adipocytes. With adipocyte differentiation, p28 is up-regulated whereas its binding partner EBI3 is significantly down-regulated. Moreover, we report that both genes are highly induced with inflammatory stress in PAs and ADs with exception of p28 which shows refractory to the inflammatory stress with adipocyte differentiation. Importantly, IL-27, but not IL-12 nor IL-23, is secreted from PAs and ADs in response to inflammation. These findings suggest that IL-27 potentially plays an important role in obesity-induced inflammation and adipocytes can be a major source for this cytokine to regulate this process. It is important to note that IL-12 and IL-23 secretion from adipocytes in culture media was undetectable using highly sensitive and specific ELISA even cells had been exposed to TNFα and LPS, despite the large increase in mRNA levels induced by these stimulations.

The interaction between the cytokine cascade networks operating within AT is an important mechanism mediating linking obesity to inflammatory diseases. It is evident that cellular cross-talk exists between adipocytes, preadipocytes, and macrophages (i.e.,

resident and invading) in the tissue. These paracrine/autocrine interactions are a crucial factor in AT function. As we found secretion of IL-27 and expression of IL-27 receptors from both PAs and ADs, we hypothesized that IL-27 acts as an inflammatory mediator in adipocytes in a paracrine/autocrine manner. As IL-27 has been reported that it serves as two distinct functions for regulation of the immune responses (21,22), we showed two contrary functions of IL-27 on adipocytes based on conditions of inflammatory state. At the normal condition, IL-27 acts as an anti-inflammatory cytokine by reducing expression of a number of pro-inflammatory genes. In contrast, IL-27 functions as a pro-inflammatory cytokine by exaggerating expression of the pro-inflammatory gene when we introduce pro-inflammatory environment by adding TNFα. Together, these findings suggest that IL-27 may play a protective role in obesity-associated inflammatory diseases. On the other hand, once AT gets inflamed, IL-27 exacerbates inflammation and negatively regulates AT metabolism and function.

In summary, this study provides the first comprehensive and systematic analysis of the IL-12 family cytokines under conditions of obesity in major insulin responsive tissues involved in metabolic homeostasis. Data presented here demonstrate that IL-12 family cytokines and receptors are divergently regulated in a tissue-specific manner under conditions of obesity. Moreover, the regulation of IL-27 cytokine in WAT is dependent on the developmental stage of obesity as well as the inflammatory progression associated with obesity. Our data further show that IL-27 is highly regulated and secreted from cultured adipocytes and plays an important role in regulating adipocyte inflammation. These data collectively suggest that divergent regulation of IL-12 family cytokines, particularly IL-27 may contribute to the obesity-associated phenomenon of inflammation in metabolic tissues.

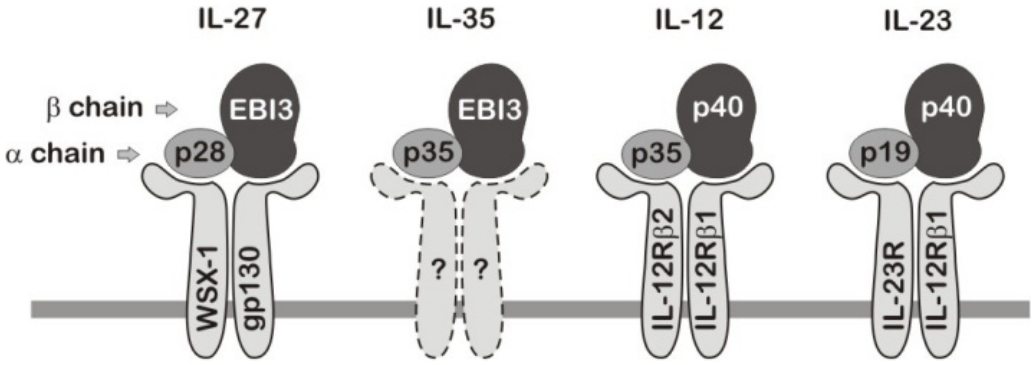

Figure 2.1. IL-12 family cytokines. IL-12 family cytokines are composed of shared alpha and beta chain subunits that dimerize to form IL-27, IL-35, IL-12, and IL-23. Each cytokine signals through unique heterodimeric cell surface receptors. Receptor composition for the newest member of the family, IL-35, has yet to be determined.

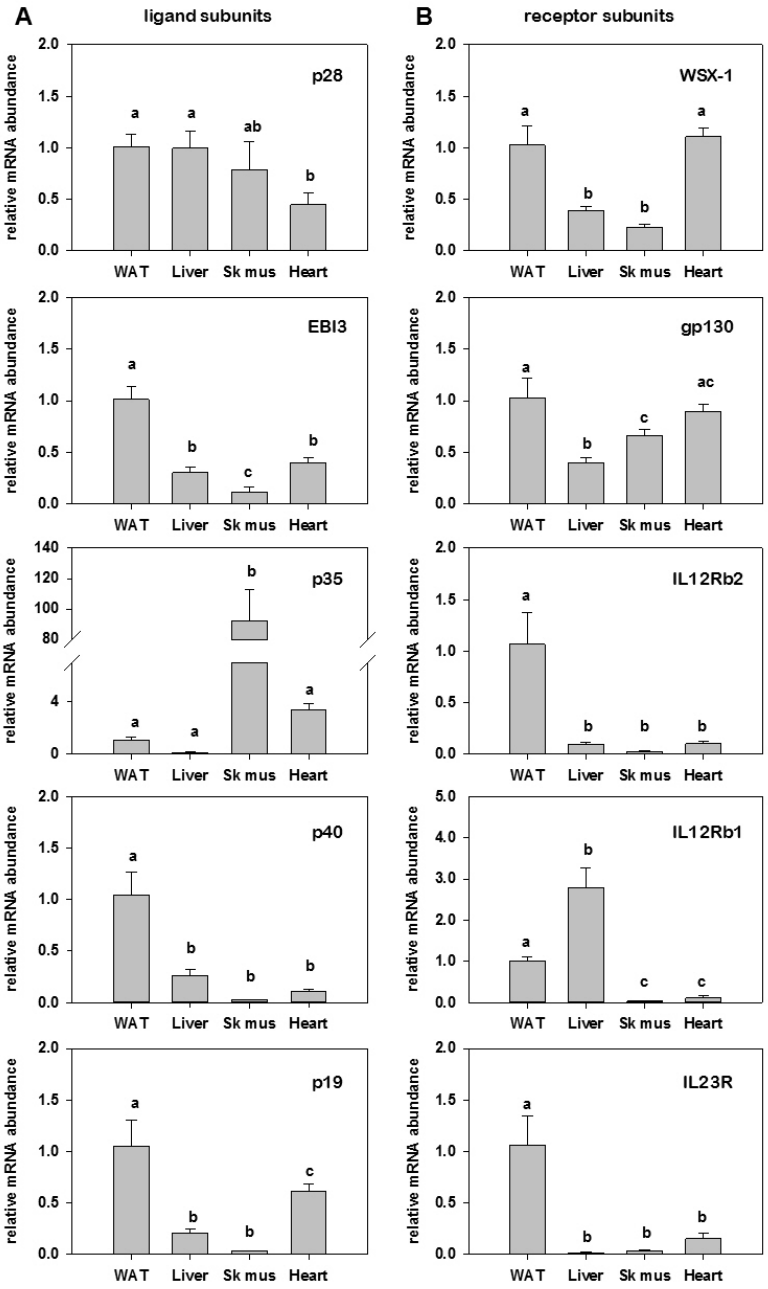

Figure 2.2. Relative IL-12 family cytokine and receptor gene expression across insulin-responsive tissues from lean C57BL/6J mice. Relative mRNA abundance of ligand (A) and receptor (B) subunits was determined by qRT-PCR from total RNA extracted from WAT, liver, skeletal muscle, and heart of 10 wk old lean C57BL/6J mice. Data were normalized to 18S rRNA and expressed as fold differences relative to WAT. Statistical differences were determined by ANOVA, with Tukey's *post-hoc* analysis performed when the *p*-value for the respective parameter was statistically significant ($p < 0.05$).

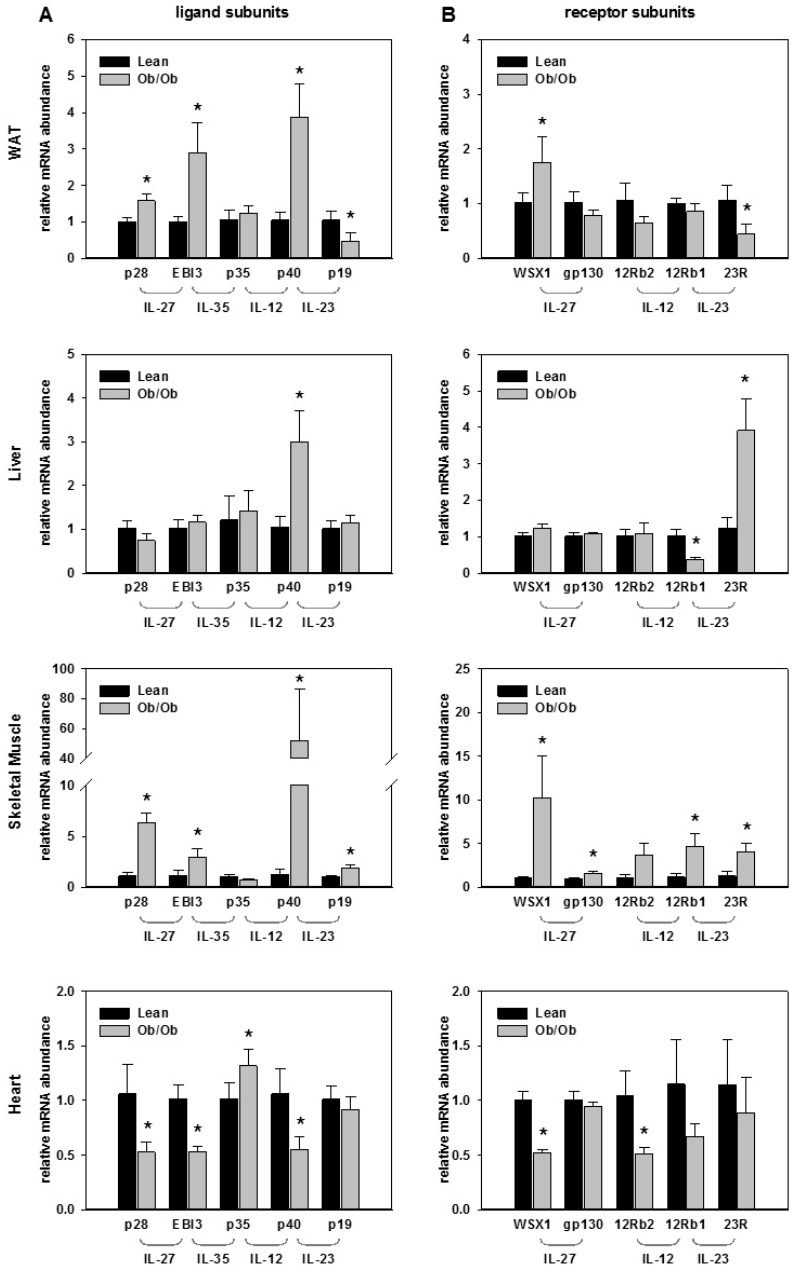

Figure 2.3. Relative IL-12 family cytokine and receptor gene expression within insulin-responsive tissues comparing lean and obese Ob/Ob mice. Relative mRNA abundance of ligand (A) and receptor (B) subunits was determined by qRT-PCR from total RNA extracted from WAT, liver, skeletal muscle and heart of 10 wk old lean and leptin-deficient (ob/ob) mice. Data were normalized to 18S rRNA and relative abundance determined for each cytokine and receptor where obese values were expressed as fold-differences relative to lean within each tissue. Differences in gene expression between lean and obese animals were determined via student's *t*-test where a *p*-value of <0.05 was considered significant.

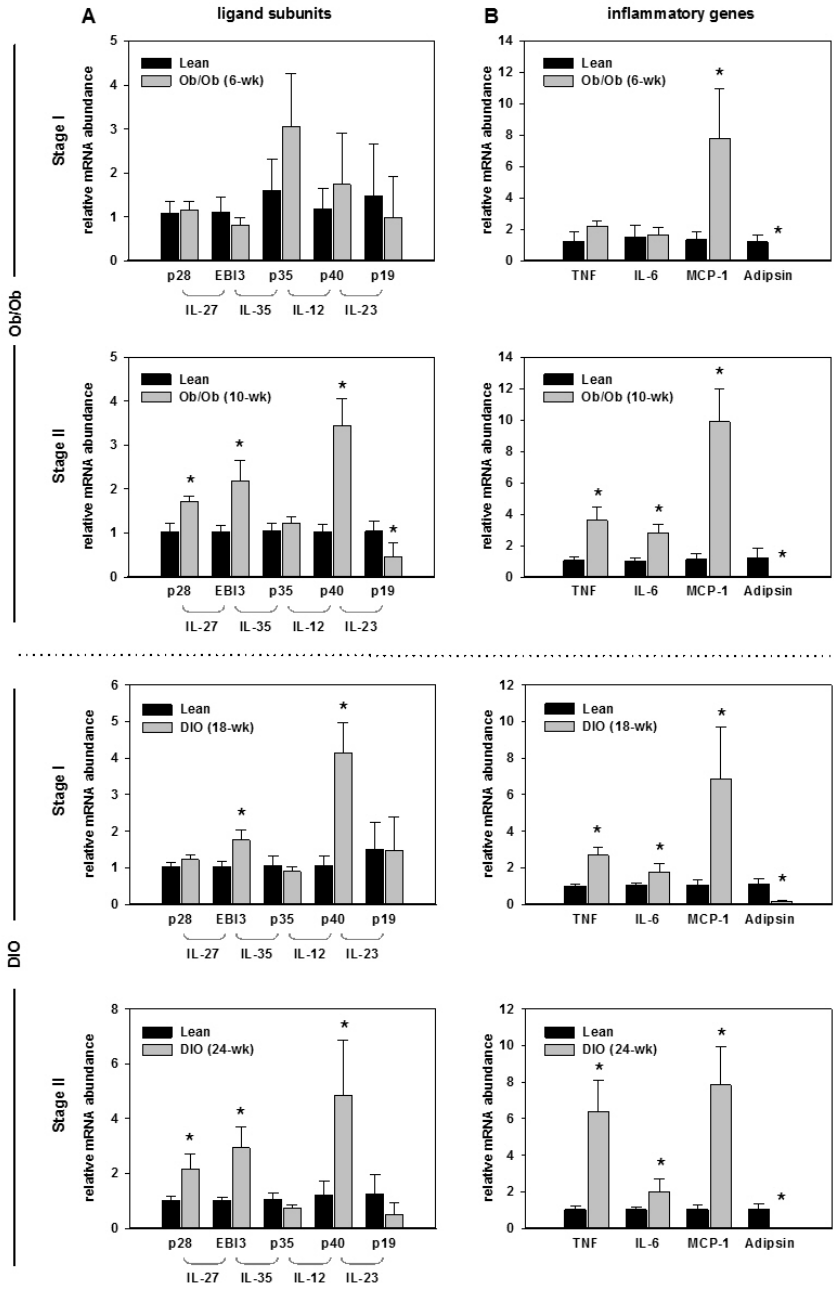

Figure 2.4. Relative IL-12 family cytokine gene expression in WAT during the progressive development of genetic and diet-induced obesity. Relative mRNA abundance of IL-12 family cytokine (A) and inflammatory genes (B) was determined by qRT-PCR from total RNA extracted from WAT at progressive stages of obesity from ob/ob mice as well as mice subject to DIO. Data were normalized to 18S rRNA and relative abundance determined for each cytokine where obese values were expressed as fold-differences relative to lean within each group. Differences in gene expression between lean and obese animals were determined via student's *t*-test where a *p*-value of <0.05 was considered significant.

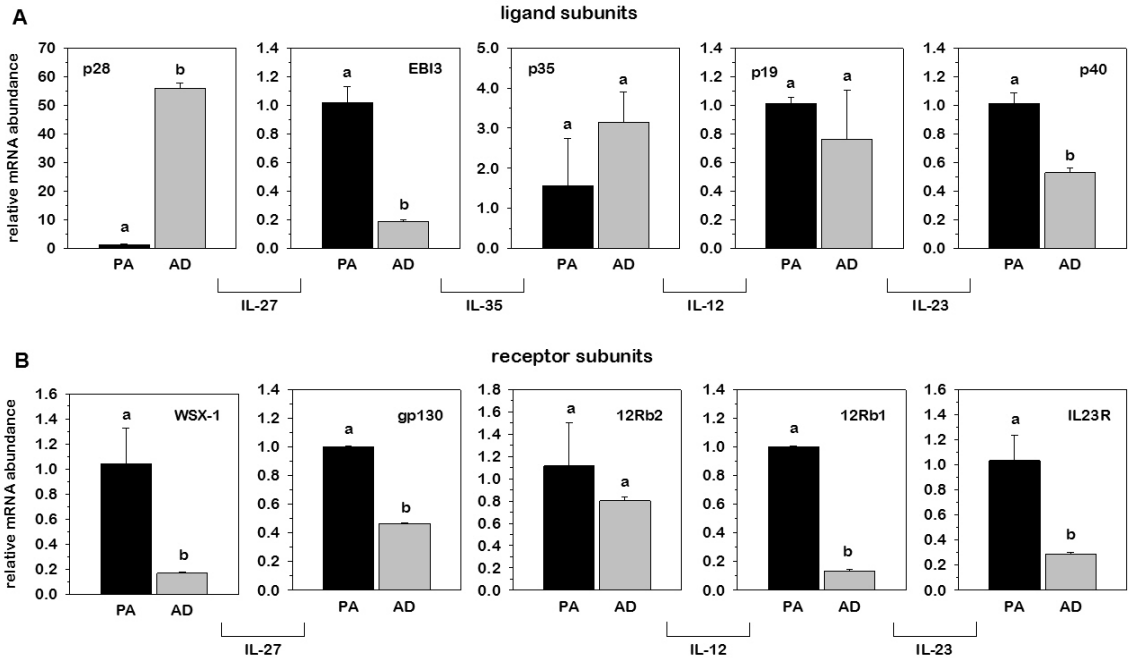

Figure 2.5. Relative IL-12 family cytokine and receptor gene expression in 3T3-L1 adipocytes comparing undifferentiated PA and mature AD mRNA abundance. Relative mRNA abundance was determined by qRT-PCR from total RNA extracted for undifferentiated 3T3-L1 preadipocytes (PA) and mature 3T3-L1 adipocytes (AD). Data were normalized to 18S rRNA and relative abundance determined for each phenotype where AD values were expressed as fold-differences relative to PA. Differences in gene expression between cell phenotypes were determined via student's *t*-test where a *p*-value of <0.05 was considered significant.

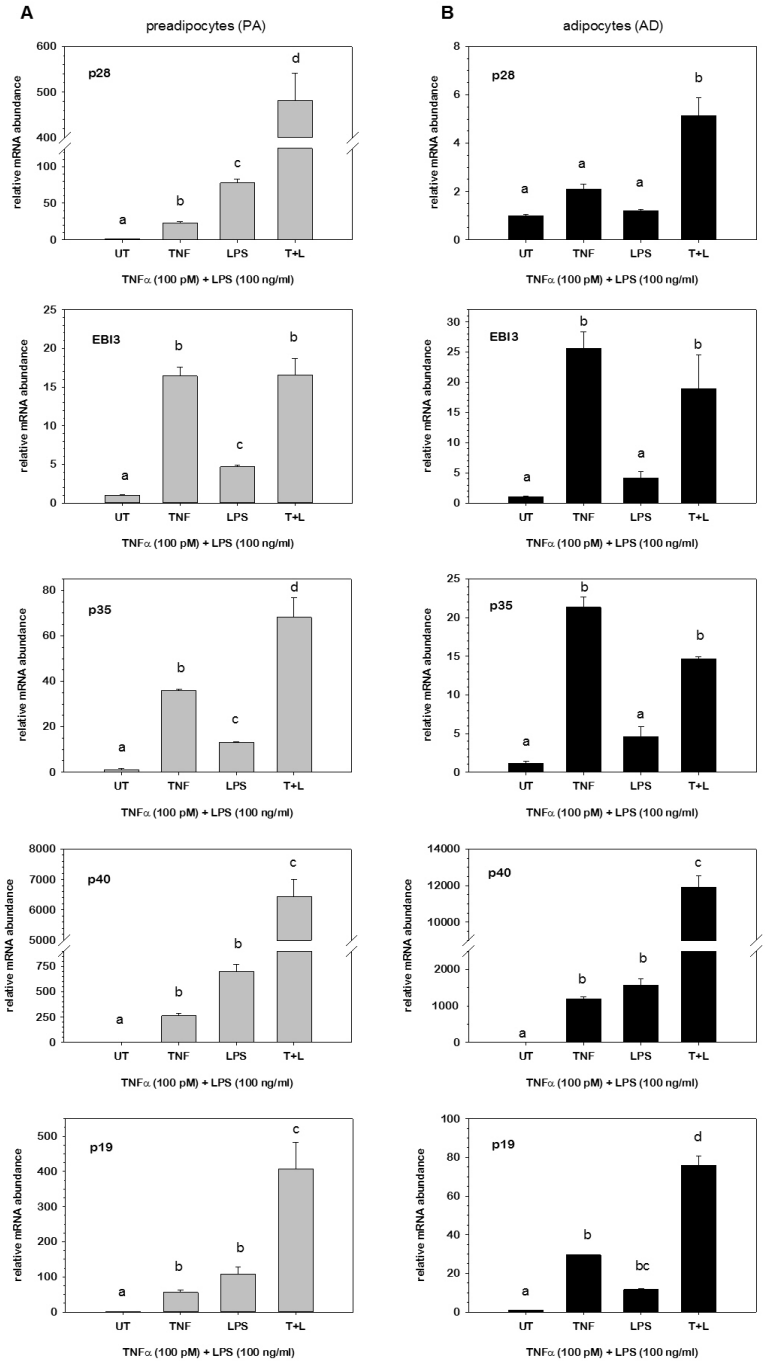

Figure 2.6. Relative IL-12 family cytokine gene expression in PAs and ADs under conditions of inflammatory stress. Relative mRNA abundance of IL-12 family cytokines was determined by qRT-PCR from total RNA extracted for 3T3-L1 PA and AD at 2 hr or 12 hr post-stimulation with 100 pM TNFα, 100 ng/ml LPS, or in combination. Data were normalized to 18S rRNA and expressed as fold differences relative to untreated (UT) controls. Statistical differences were determined by ANOVA, with Tukey's *post-hoc* analysis performed when the *p*-value for the respective parameter was statistically significant ($p < 0.05$).

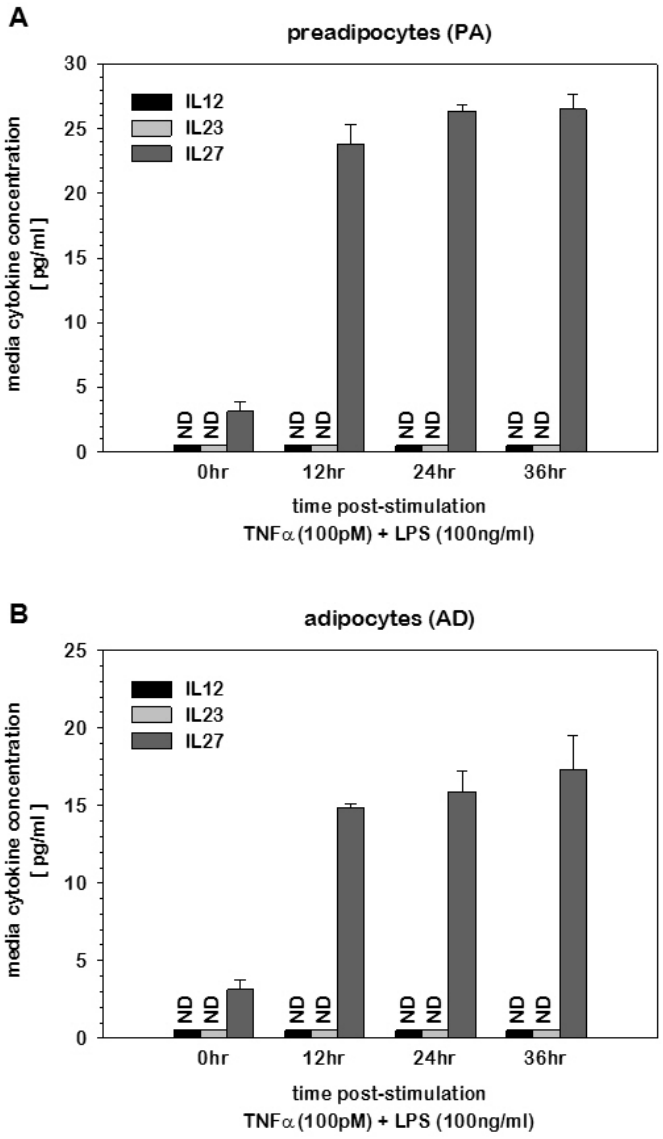

Figure 2.7. Accumulation of IL-12 family cytokines in culture media of PAs and ADs under conditions of inflammatory stress. Concentration of IL-12, IL-23 and IL-27 was determined by ELISA in cell media over time following TNFα (100 pM) and LPS (100 ng/ml) stimulation of 3T3-L1 undifferentiated preadipocytes (PA) and mature adipocytes (AD). Values not detected (ND) were indicated.

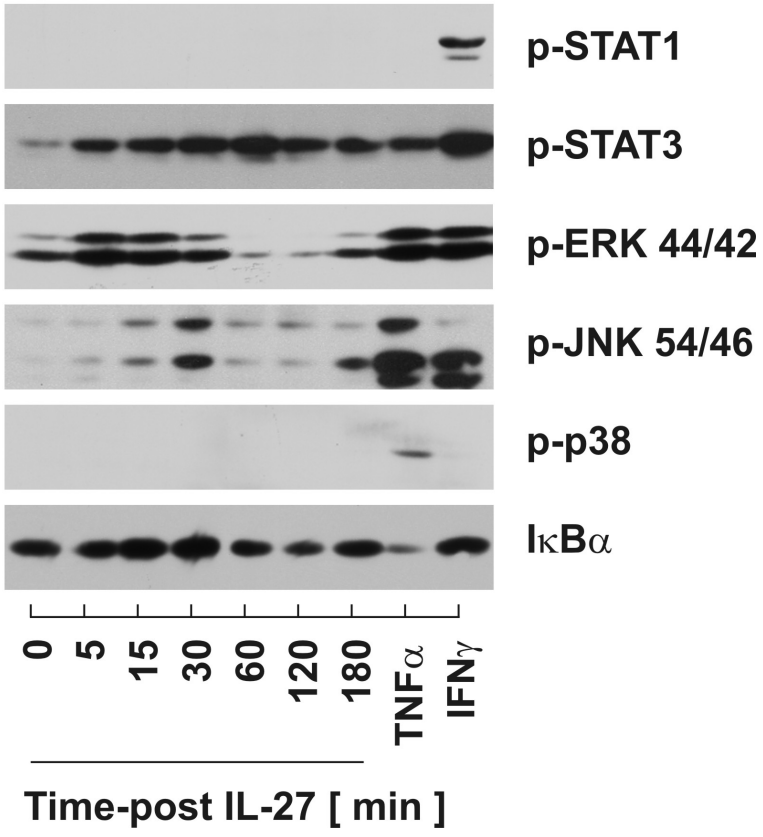

Figure 2.8. Effect of IL-27 stimulation on inflammatory signaling pathways. Signaling pathways were evaluated by immunoblotting cell lysates collected from 3T3-L1 preadipocytes (PA) over time following 50 nM IL-27 stimulation. TNFα (100 pM) and IFNγ (20 ng/ml) were included as positive controls for individual pathways.

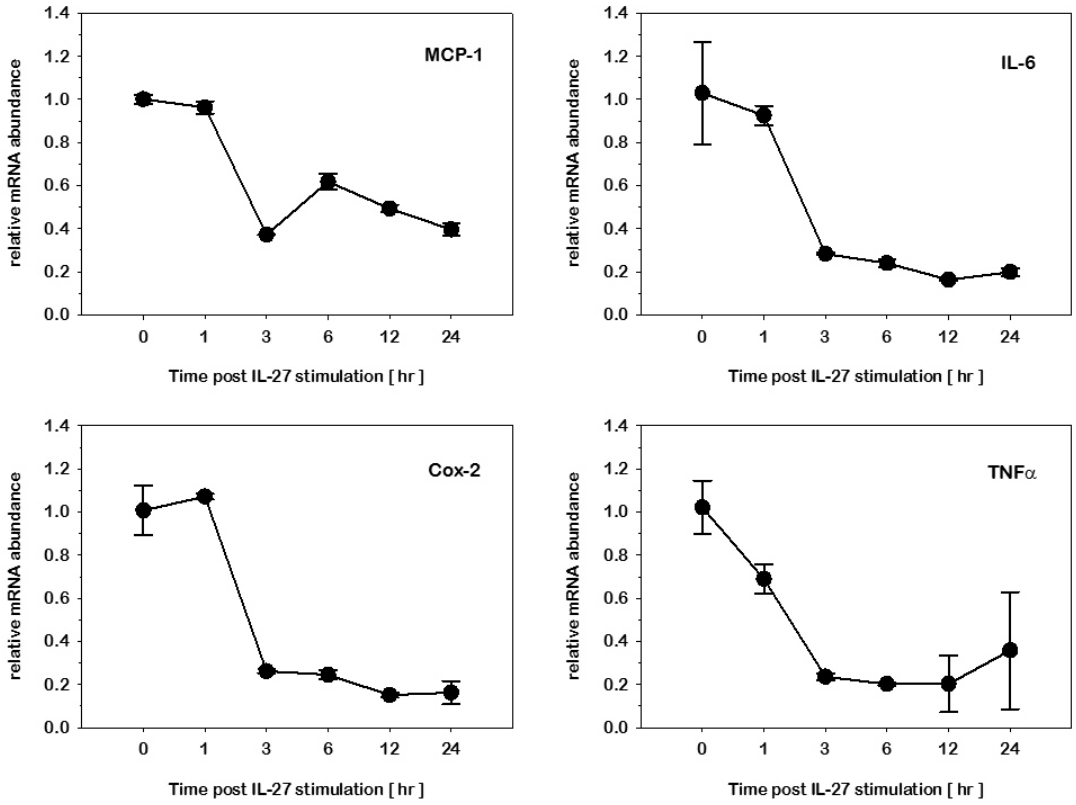

Figure 2.9. Effect of IL-27 stimulation on basal inflammatory gene expression. Relative mRNA abundance was determined by qRT-PCR over time for each inflammatory gene following 50 nM IL-27 stimulation of 3T3-L1 preadipocytes (PA). Data were normalized to 18S rRNA and relative abundance for each time point was expressed as fold-differences relative to levels observed prior to stimulation (0 hr).

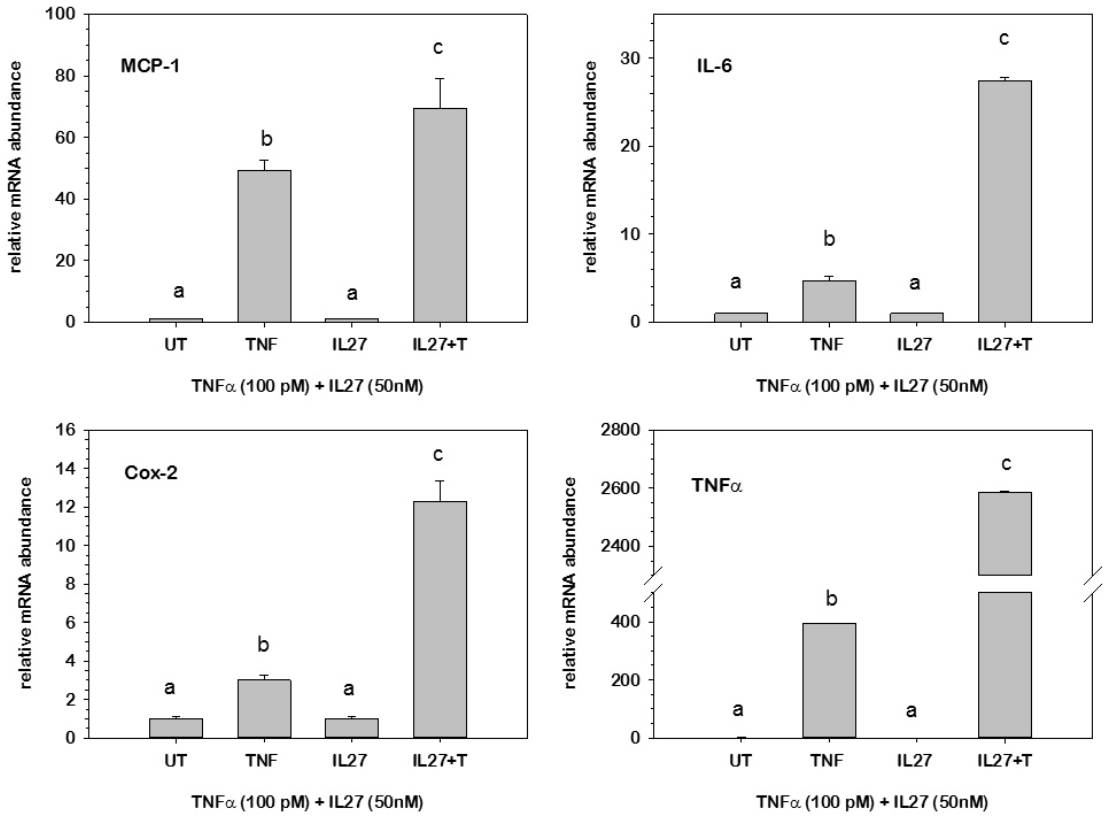

Figure 2.10. Effect of IL-27 stimulation on cytokine-mediated inflammatory gene expression. Relative mRNA abundance of specified inflammatory genes was determined by qRT-PCR from total RNA extracted from 3T3-L1 preadipocytes (PA) at 2 hrs post-stimulation with 100 pM TNFα or 50 nM IL-27 or in combination. Data were normalized to 18S rRNA and expressed as fold differences relative to untreated (UT) controls and IL-27 for TNFα stimulation and IL-27+TNFα stimulation, respectively. Statistical differences were determined by ANOVA, with Tukey's *post-hoc* analysis performed when the *p*-value for the respective parameter was statistically significant ($p < 0.05$).

Reference List

1. Haslam DW, James WP. Obesity. Lancet. 2005 Oct 1;366:1197-209.

2. Caterson ID, Gill TP. Obesity: epidemiology and possible prevention. Best Pract Res Clin Endocrinol Metab. 2002 Dec;16:595-610.

3. Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol. 2008 May;9:367-77.

4. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993 Jan 1;259:87-91.

5. Maury E, Noel L, Detry R, Brichard SM. In vitro hyperresponsiveness to tumor necrosis factor-alpha contributes to adipokine dysregulation in omental adipocytes of obese subjects. J Clin Endocrinol Metab. 2009 Apr;94:1393-400.

6. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature. 1997 Oct 9;389:610-4.

7. Sartipy P, Loskutoff DJ. Monocyte chemoattractant protein 1 in obesity and insulin resistance. Proc Natl Acad Sci U S A. 2003 Jun 10;100:7265-70.

8. Baffy G. Kupffer cells in non-alcoholic fatty liver disease: the emerging view. J Hepatol. 2009 Jul;51:212-23.

9. Cai D, Yuan M, Frantz DF, Melendez PA, Hansen L, Lee J, Shoelson SE. Local and systemic insulin resistance resulting from hepatic activation of IKK-beta and NF-kappaB. Nat Med. 2005 Feb;11:183-90.

10. Carey AL, Bruce CR, Sacchetti M, Anderson MJ, Olsen DB, Saltin B, Hawley JA, Febbraio MA. Interleukin-6 and tumor necrosis factor-alpha are not increased in patients with Type 2 diabetes: evidence that plasma interleukin-6 is related to fat mass and not insulin responsiveness. Diabetologia. 2004 Jun;47:1029-37.

11. Gregor MF, Hotamisligil GS. Inflammatory mechanisms in obesity. Annu Rev Immunol. 2011 Apr 23;29:415-45.

12. Ouwens DM, Sell H, Greulich S, Eckel J. The role of epicardial and perivascular adipose tissue in the pathophysiology of cardiovascular disease. J Cell Mol Med. 2010 Sep;14:2223-34.

13. Winkler G, Dworak O, Salamon F, Salamon D, Speer G, Cseh K. Increased interleukin-12 plasma concentrations in both, insulin-dependent and non-insulin-dependent diabetes mellitus. Diabetologia. 1998 Apr;41:488.

14. Wegner M, Winiarska H, Bobkiewicz-Kozlowska T, Dworacka M. IL-12 serum levels in patients with type 2 diabetes treated with sulphonylureas. Cytokine. 2008 Jun;42:312-6.

15. Wen Y, Gu J, Li SL, Reddy MA, Natarajan R, Nadler JL. Elevated glucose and diabetes promote interleukin-12 cytokine gene expression in mouse macrophages. Endocrinology. 2006 May;147:2518-25.

16. Surendar J, Mohan V, Rao MM, Babu S, Aravindhan V. Increased levels of both Th1 and Th2 cytokines in subjects with metabolic syndrome (CURES-103). Diabetes Technol Ther. 2011 Apr;13:477-82.

17. Goriely S, Goldman M. Interleukin-12 family members and the balance between rejection and tolerance. Curr Opin Organ Transplant. 2008 Feb;13:4-9.

18. Merberg DM, Wolf SF, Clark SC. Sequence similarity between NKSF and the IL-6/G-CSF family. Immunol Today. 1992 Feb;13:77-8.

19. Collison LW, Vignali DA. Interleukin-35: odd one out or part of the family? Immunol Rev. 2008 Dec;226:248-62.

20. Trinchieri G. Interleukin-12 and the regulation of innate resistance and adaptive immunity. Nat Rev Immunol. 2003 Feb;3:133-46.

21. Hunter CA. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. Nat Rev Immunol. 2005 Jul;5:521-31.

22. Kastelein RA, Hunter CA, Cua DJ. Discovery and biology of IL-23 and IL-27: related but functionally distinct regulators of inflammation. Annu Rev Immunol. 2007;25:221-42.

23. Yoshimura T, Takeda A, Hamano S, Miyazaki Y, Kinjyo I, Ishibashi T, Yoshimura A, Yoshida H. Two-sided roles of IL-27: induction of Th1 differentiation on naive CD4+ T cells versus suppression of proinflammatory cytokine production including IL-23-induced IL-17 on activated CD4+ T cells partially through STAT3-dependent mechanism. J Immunol. 2006 Oct 15;177:5377-85.

24. Kempe S, Heinz P, Kokai E, Devergne O, Marx N, Wirth T. Epstein-barr virus-induced gene-3 is expressed in human atheroma plaques. Am J Pathol. 2009 Jul;175:440-7.

25. Wirtz S, Becker C, Fantini MC, Nieuwenhuis EE, Tubbe I, Galle PR, Schild HJ, Birkenbach M, Blumberg RS, Neurath MF. EBV-induced gene 3 transcription is induced by TLR signaling in primary dendritic cells via NF-kappa B activation. J Immunol. 2005 Mar 1;174:2814-24.

26. Sumarac-Dumanovic M, Stevanovic D, Ljubic A, Jorga J, Simic M, Stamenkovic-Pejkovic D, Starcevic V, Trajkovic V, Micic D. Increased activity of interleukin-23/interleukin-17 proinflammatory axis in obese women. Int J Obes (Lond). 2009 Jan;33:151-6.

27. Cousin B, Munoz O, Andre M, Fontanilles AM, Dani C, Cousin JL, Laharrague P, Casteilla L, Penicaud L. A role for preadipocytes as macrophage-like cells. FASEB J. 1999 Feb;13:305-12.

28. Febbraio MA. gp130 receptor ligands as potential therapeutic targets for obesity. J Clin Invest. 2007 Apr;117:841-9.

29. White UA, Stewart WC, Mynatt RL, Stephens JM. Neuropoietin attenuates adipogenesis and induces insulin resistance in adipocytes. J Biol Chem. 2008 Aug 15;283:22505-12.

30. Song H, Lim H. Evidence for heterodimeric association of leukemia inhibitory factor (LIF) receptor and gp130 in the mouse uterus for LIF signaling during blastocyst implantation. Reproduction. 2006 Feb;131:341-9.

31. Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. Nat Rev Immunol. 2008 Dec;8:923-34.

32. Keller MP, Attie AD. Physiological insights gained from gene expression analysis in obesity and diabetes. Annu Rev Nutr. 2010 Aug 21;30:341-64.

33. Djian P, Phillips M, Green H. The activation of specific gene transcription in the adipose conversion of 3T3 cells. J Cell Physiol. 1985 Sep;124:554-6.

34. Morrison RF, Farmer SR. Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. J Biol Chem. 1999 Jun 11;274:17088-97.

35. Ferguson BS, Nam H, Hopkins RG, Morrison RF. Impact of reference gene selection for target gene normalization on experimental outcome using real-time qRT-PCR in adipocytes. PLoS One. 2010;5:e15208.

36. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25:402-8.

37. Cook KS, Min HY, Johnson D, Chaplinsky RJ, Flier JS, Hunt CR, Spiegelman BM. Adipsin: a circulating serine protease homolog secreted by adipose tissue and sciatic nerve. Science. 1987 Jul 24;237:402-5.

38. Johnson PR, Spiegelman B, Rosen B, Turkenkopf I, Ree H, Greenwood MR. Reduced adipsin mRNA and circulating adipsin protein are modulated by adrenal steroids in obese Zucker rats. Am J Physiol. 1990 Jul;259:R184-R188.

39. Li Z, Oben JA, Yang S, Lin H, Stafford EA, Soloski MJ, Thomas SA, Diehl AM. Norepinephrine regulates hepatic innate immune system in leptin-deficient mice with nonalcoholic steatohepatitis. Hepatology. 2004 Aug;40:434-41.

40. Li Z, Soloski MJ, Diehl AM. Dietary factors alter hepatic innate immune system in mice with nonalcoholic fatty liver disease. Hepatology. 2005 Oct;42:880-5.

41. Kim SJ, Choi Y, Choi YH, Park T. Obesity activates toll-like receptor-mediated proinflammatory signaling cascades in the adipose tissue of mice. J Nutr Biochem. 2011 Mar 15.

42. Gomez-Merino D, Drogou C, Guezennec CY, Chennaoui M. Effects of chronic exercise on cytokine production in white adipose tissue and skeletal muscle of rats. Cytokine. 2007 Oct;40:23-9.

43. Yoshida H, Miyazaki Y. Regulation of immune responses by interleukin-27. Immunol Rev. 2008 Dec;226:234-47.

44. Hamano S, Himeno K, Miyazaki Y, Ishii K, Yamanaka A, Takeda A, Zhang M, Hisaeda H, Mak TW, et al. WSX-1 is required for resistance to Trypanosoma cruzi infection by regulation of proinflammatory cytokine production. Immunity. 2003 Nov;19:657-67.

45. Takeda A, Hamano S, Yamanaka A, Hanada T, Ishibashi T, Mak TW, Yoshimura A, Yoshida H. Cutting edge: role of IL-27/WSX-1 signaling for induction of T-bet through activation of STAT1 during initial Th1 commitment. J Immunol. 2003 May 15;170:4886-90.

46. Yamanaka A, Hamano S, Miyazaki Y, Ishii K, Takeda A, Mak TW, Himeno K, Yoshimura A, Yoshida H. Hyperproduction of proinflammatory cytokines by WSX-1-deficient NKT cells in concanavalin A-induced hepatitis. J Immunol. 2004 Mar 15;172:3590-6.

47. Baker BJ, Park KW, Qin H, Ma X, Benveniste EN. IL-27 inhibits OSM-mediated TNF-alpha and iNOS gene expression in microglia. Glia. 2010 Jul;58:1082-93.

48. Kleemann R, Zadelaar S, Kooistra T. Cytokines and atherosclerosis: a comprehensive review of studies in mice. Cardiovasc Res. 2008 Aug 1;79:360-76.

49. Gustafson B. Adipose tissue, inflammation and atherosclerosis. J Atheroscler Thromb. 2010 Apr 30;17:332-41.

50. Stumhofer JS, Tait ED, Quinn WJ, III, Hosken N, Spudy B, Goenka R, Fielding CA, O'Hara AC, Chen Y, et al. A role for IL-27p28 as an antagonist of gp130-mediated signaling. Nat Immunol. 2010 Dec;11:1119-26.

51. Maaser C, Egan LJ, Birkenbach MP, Eckmann L, Kagnoff MF. Expression of Epstein-Barr virus-induced gene 3 and other interleukin-12-related molecules by human intestinal epithelium. Immunology. 2004 Jul;112:437-45.

52. Sonobe Y, Yawata I, Kawanokuchi J, Takeuchi H, Mizuno T, Suzumura A. Production of IL-27 and other IL-12 family cytokines by microglia and their subpopulations. Brain Res. 2005 Apr 8;1040:202-7.

53. Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. J Clin Invest. 2003 Dec;112:1785-8.

54. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. A central role for JNK in obesity and insulin resistance. Nature. 2002 Nov 21;420:333-6.

55. Strissel KJ, Stancheva Z, Miyoshi H, Perfield JW, DeFuria J, Jick Z, Greenberg AS, Obin MS. Adipocyte death, adipose tissue remodeling, and obesity complications. Diabetes. 2007 Dec;56:2910-8.

56. Xu H, Barnes GT, Yang Q, Tan G, Yang D, Chou CJ, Sole J, Nichols A, Ross JS, et al. Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. J Clin Invest. 2003 Dec;112:1821-30.

57. Shaul ME, Bennett G, Strissel KJ, Greenberg AS, Obin MS. Dynamic, M2-like remodeling phenotypes of CD11c+ adipose tissue macrophages during high-fat diet--induced obesity in mice. Diabetes. 2010 May;59:1171-81.

58. Weisberg SP, McCann D, Desai M, Rosenbaum M, Leibel RL, Ferrante AW, Jr. Obesity is associated with macrophage accumulation in adipose tissue. J Clin Invest. 2003 Dec;112:1796-808.

59. Strissel KJ, DeFuria J, Shaul ME, Bennett G, Greenberg AS, Obin MS. T-cell recruitment and Th1 polarization in adipose tissue during diet-induced obesity in C57BL/6 mice. Obesity (Silver Spring). 2010 Oct;18:1918-25.

60. Lolmede K, Duffaut C, Zakaroff-Girard A, Bouloumie A. Immune cells in adipose tissue: Key players in metabolic disorders. Diabetes Metab. 2011 Apr 18.

CHAPTER III

DIFFERENTIAL REGULATION OF IL-27 BY HISTONE DEACETYLASES DURING INFLAMMATORY STRESS IN ADIPOCYTES

Abstract

Chronic inflammation is an important element of pathogenic mechanisms linking obesity and diabetes. Interleukin-27 (IL-27) is a heterodimeric inflammatory cytokine encoded by independent p28 and EBI3 genes and plays a critical role in initiating and maintaining cell-mediated immune responses. Our previous investigations reported that IL-27 mRNA is significantly elevated in adipose tissue (AT) of genetic and diet-induced murine models of obesity. In addition, we have previously shown that cultured preadipocytes (PAs) and adipocytes (ADs) secrete IL-27 under conditions of inflammatory stress. In this study, we investigated the molecular mechanisms that regulate IL-27 in 3T3-L1 adipocytes using TNFα as a model of inflammatory stress. We report here that IL-27 genes were divergently regulated based on cell phenotype with p28 mRNA significantly more abundant in ADs compared to PAs and EBI3 mRNA significantly more abundant in PAs compared to ADs. We further report that TNFα resulted in marked induction of p28 and EBI3 gene expression, but with different kinetics where mRNA abundance peaked at 4 hrs and 12 hrs, respectively. While similar kinetics of induction were observed for EBI3 in ADs, there was no marked induction in p28 expression at any given concentration of TNFα. Pharmacological inhibition of inflammatory signaling identified NF-κB as the predominant upstream activator of both p28 and EBI3. However, the divergent regulation of IL-27 within the same cell type as well as the refractory nature of

p28 in ADs was not due to differences in NF-κB signaling, as this pathway was similarly activated in PAs and ADs. As a plausible mechanism regarding differences in gene regulation, we further identified histone deacetylase (HDAC) activity was necessary for TNFα-induced p28 in PAs, but not ADs. Conversely, EBI3 expression in response to TNFα was completely independent with HDAC activity. Collectively, these findings suggest a role for HDACs in the divergent regulation of IL-27 gene expression in response to inflammatory stress and adipocyte differentiation.

Introduction

Obesity is a major risk factor for cardiovascular disease, stroke, and type 2 diabetes. Early studies have revealed an important role for AT in the regulation and control of whole-body metabolic homeostasis in normal and diseased states. Furthermore, evidence indicates that cellular and molecular mechanisms involved in AT metabolism are altered by obesity, leading to insulin resistance (IR) (1,2). It is now well-established that chronic inflammation, originating in AT, is an important element of pathogenic mechanisms linking obesity and diabetes (3,4). Molecular mechanisms that underlie the initiation of AT inflammation during the onset of obesity include synthesis and secretion of various chemokines, such as monocyte chemoattractant protein-1 (MCP-1), that enhance AT accumulation of macrophages leading to further inflammatory processes mediating IR (5). While it is now evident that chronic, low-grade inflammation is highly associated with obesity-induced inflammation and IR, key inflammatory mediators that contribute to this process have not been fully elucidated.

IL-27 is a member of IL-12 family cytokines that play crucial roles during inflammatory processes. This cytokine is composed of two heterodimeric subunits (i.e., p28 and EBI3), and initiates its actions through binding to a heterodimeric cell-surface receptor composed of WSX-1 and gp130, where gp130 is required for both ligand binding and signal transduction (6). While early literature suggested that IL-27 expression is restricted to myeloid cells including monocytes, monocyte-derived dendritic cells (DCs), and macrophages (7), more recent studies have also reported regulation of IL-27 in other, traditionally non-immune cell types. To this point, EBI3 and p28 have been shown to be expressed in trophoblasts throughout human pregnancy (8) and highly expressed in vascular smooth muscle cells and endothelial cells in response to pro-inflammatory stimuli (i.e., TNFα or IFNγ) (9,10). Similarly, previous reports from our lab have demonstrated that IL-27 expression was higher in AT of genetic and diet-induced murine models of obesity and that IL-27 was secreted from cultured adipocytes under conditions of inflammatory stress. As evidence has established a mechanistic role for gp130 cytokines in obesity-induced IR (11-13), these findings support a role for IL-27 in the mechanisms linking excess nutrient intake to AT inflammation and IR.

Activation of nuclear factor kappa B (NF-κB) signaling events by various inflammatory stimuli largely contribute to the transcriptional regulation of IL-27 in classical immune cells (14,15). NF-κB is a family of transcription factors composed of five subunits: p50, p52, p65 (RelA), p62 (RelB), and c-Rel, which form homodimers and/or heterodimers to regulate inflammatory gene expression. Canonically, NF-κB subunits are held in the cytosol under basal conditions and bounded a group of proteins known as inhibitors of NF-κB (IκBs). Upstream IκB kinases (IKKs) are activated by inflammatory stimuli leading to the phosphorylation and subsequent degradation of IκBs

and the release of NF-κB subunits that translocate to the nucleus where they act as a transcription factors to promote inflammatory gene expression (16,17). In addition to compartmental considerations, NF-κB transcriptional activity is dependent on post-translational modifications, such as acetylation, that promote interaction with co-activators and co-repressors (16,18,19). While co-activators, such as CREB binding protein (CBP) and the steroid receptor coactivator (SRC) family play an important role in activation of NF-κB mediated transcriptional activity (20-23), co-repressors, such as histone deacetylases (HDACs) and nuclear receptor corepressor (N-CoR) negatively regulate NF-κB dependent gene expression (23,24). In contrast to their inhibitory role in the transcriptional complex, recent reports have demonstrated that HDACs also promote NF-κB activity at multiple steps along the pathway that are critical for nuclear translocation and promoter interaction (25,26) as well as IκBα degradation (27). Accordingly, HDACs has been deemed anti-inflammatory as treatment with HDAC inhibitors negatively regulate NF-κB-dependent transcription by blocking translocation and DNA binding as well as interfering with RNA polymerase II recruitment (28,29).

In the present study, we investigated the role of NF-κB signaling on epigenetic regulation of IL-27 in adipocytes in response to inflammation. We showed that divergent regulation of IL-27 genes, p28 and EBI3, involved HDAC-dependent and -independent mechanisms downstream of NF-κB signaling and that the refractory nature of p28 in ADs was not due to differences in upstream signaling pathways as they were similarly activated in PAs and ADs. Collectively, our findings support a regulatory role for HDACs that is both gene and cell-type specific, potentially contributing to the refractory nature of p28 as PAs mature into ADs as well as the exacerbated response of PAs to inflammatory stress.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DMEM), calf bovine serum (CS), Trypsin-EDTA, and recombinant murine TNFα were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from HyClone. The following antibodies were used for immunoblot analysis: phospho-ERK (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), IκBα, phospho-IKKα (Ser176) /IKKβ (Ser 177), α-tubulin (Cell Signaling), NFκBp65 (Santa Cruz), Nucleoporin p62 (BD transduction). Trichostatin A (TSA), sodium butyrate (NaB), and nicotinamide (NAM) were obtained from Sigma. Pharmacological inhibitors of ERK (U0126), JNK (SP600125), p38 (SB203580) were purchased from CalBiochem. NF-κB inhibitor (helenalin) was obtained from Biomol. Enhanced chemiluminescence (ECL) reagents were obtained from Perkin-Elmer Life Sciences. All TaqMan primer probes used in this study were purchased from Applied Biosystems.

Cell culture. The murine 3T3-L1 cell line was purchased from Howard Green, Harvard Medical School (30). Cells were propagated in DMEM supplemented with 10% CS until reaching density-induced arrest as previously described (31). At 2 days post-confluence, growth medium was replaced with DMEM supplemented with 10% FBS, 0.5mM 1-methy-3-isobutylxanthane, 1µM dexamethasone, and 1.7 µM insulin (MDI) for 2 days. Subsequently, cells were cultured in DMEM supplemented only with 10% FBS over the following six days as PAs differentiated into mature ADs. Throughout the study, 'time 0' refers to density arrested cells immediately before the addition of MDI to the culture medium. Experiments described herein were conducted in density-arrested day 0

(d0) PAs or day 8 (d8) ADs. All experiments were repeated 2-3 times to validate results and ensure reliability.

Immunoblotting. Cell monolayers were washed with phosphate-buffer saline (PBS) and scraped into ice-cold lysis buffer containing 0.1 M Tris (pH 7.4), 150 mM NaCl, 10% sodium dodecyl sulfate (SDS), 1% Triton X, 0.5% Nonidet P-40 (NP40), 1 mM EDTA, 1 mM EGTA. Phosphatase inhibitors (20 mM β-glycerophosphate, 10 mM sodium fluoride and 2 μM sodium orthovanadate) and protease inhibitors (0.3 μM aprotinin, 21 μM leupetin, 1 μM pepstatin, 50 μM phenanthroline, 0.5 μM phenylmethylsulfonyl fluoride) were added to lysis buffer immediately prior to cell harvest. Cell lysates were sonicated and centrifuged (13,000x*g*, 10 min, 4ºC), and the supernatant transferred to a fresh tube. Protein content was determined by bicinchoninic acid (BCA) procedures according to manufacturer's (Pierce, Rockford, IL) instructions. Equal amounts of whole cell lysate protein were separated by SDS-PAGE electrophoresis. Cell lysates were mixed with loading buffer containing 0.25 M Tris (pH6.8), 4% SDS, 10% glycerol, 0.01% bromophenol blue, and 10% dithiothreitol, then heated at 80ºC for 5 min prior to electrophoresis. Proteins were resolved on SDS-polyacylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore corp., Billerica, MA). After transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4ºC. Membranes were subsequently probed with horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature. Membranes were immersed in ECL and data visualized by autoradiography using CL-XPosure film (Pierce).

Real-time qRT-PCR. Total RNA was extracted and genomic DNA contamination was removed using the RNeasy Plus Mini Kit (Qiagen, Valencia, Ca), according to manufacturer's protocol. Total RNA was quantified with a Nanodrop ND-1000 spectrophotometer and reverse transcribed to cDNA in a 10 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems). The reverse transcription (RT) master mix containing RT buffer, deoxyribonucleotide triphosphate (dNTP) mix, RT random primers, RNase inhibitor (1.0 U/µl), and MultiScribe RT was added to 1 µg RNA and RNase-free water. Reverse transcription reaction conditions followed the protocol (25°C for 10 min, 37°C for 120 min, 85°C for 5 sec, followed by 4°C indefinitely/ RT complete) and utilized the Gene Amp PCR System 9700 thermal cycler (Applied Biosystems) for cDNA synthesis.

PCR amplification was run utilizing the 7500 fast system (Applied Biosystems, Inc.) that consisted of enzyme activation at 95°C for 20 sec, followed by 40 cycles of denaturation at 95°C for 3 sec combined with annealing/extension at 60°C for 30 sec. All data were analyzed with the ABI 7500 real time PCR system. All TaqMan primer probes used in this study were also purchased from ABI. Data were recorded and analyzed with ABI Sequence Detector Software and graphs visualized with SigmaPlot software. All data were presented as mean ± standard error of the mean (SEM) and representative of duplicate determinations. Data were normalized to 18S and measured as relative differences using the $2^{-\Delta\Delta CT}$ method as previously described (32,33).

Statistical analyses were conducted using SPSS v18. Phenotypic differences were determined via student's *t*-test where *p*-value of <0.05 was considered significant.

Inhibitor data were analyzed using analysis of variance, with Tukey's *post-hoc* analysis conducted to assess differences from controls (TNFα) when $p<0.05$.

Nuclear/cytosolic fractionation. Cell were washed with PBS and incubated with ice cold isotonic buffer containing 20 mM Tris, pH 7.4, 125 mM NaCl, 1 mM EGTA, 1 mM EDTA, and 1mM $MgCl_2$, for 10 min on ice. The buffer was supplemented with fleshly prepared 0.1% NP40, and phosphatase/protease inhibitors as described above. Following detergent solubilization, cytosolic fractions were collected from the cell monolayer and clarified by centrifugation (13,000x*g*, 5 min, 4°C). Intact nuclei were subsequently collected in PBS and gently pelleted by centrifugation (300x*g*, 3 min, 4°C). Nuclear proteins were extracted in ice cold buffer containing 20 mM Tri (pH 7.4), 1% Triton X, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA supplemented with protease/phosphatase inhibitors and frozen at -80°C. Nuclear fractions were subsequently thawed on ice, sonicated, passes through a 21 gauge needle to shear DNA, and clarified by centrifugation (13,000x*g*, 10 min,4°C).

Results

Divergent expression of IL-27 between PAs and ADs in response to TNFα. We previously demonstrated that IL-27 was elevated in animal models of obesity with documented inflammation and that PAs and ADs secreted IL-27 protein in response to inflammatory stress. As PAs and ADs are responsive to inflammatory stress, we examined the effect of TNFα on the regulation of IL-27 in these functionally diverse cell types of adipocyte lineage. Phenotypic comparisons of IL-27 was determined using the 3T3-L1 murine cell line that yielded quiescent, undifferentiated PAs prior to

differentiation and greater than 90% ADs following differentiation. Furthermore, this well-established cell line is devoid of other cell types (e.g., macrophages) providing ideal conditions for cell type-specific analyses. As an initial screen, cells were differentiated for 8 days to yield mature ADs and IL-27 mRNA expression compared to undifferentiated, density-arrested PAs of similar passage. As illustrated in Fig.3.1A, IL-27 subunit genes displayed phenotypic differences in expression where p28 mRNA was significantly elevated in ADs compared to PAs and, conversely, EBI3 mRNA was conversely elevated in PAs compared to ADs. Following this initial phenotypic comparison, PAs and ADs were stimulated concurrently with 100 pM TNFα and total RNA harvested over time to assess mRNA expression of IL-27 by qRT-PCR. This concentration of TNFα was selected as it closely approximates the concentration of TNFα in human and animal models of obesity and has been shown to suppress insulin signaling in culture (34,35). As illustrated in Fig.3.1B, IL-27 subunits, p28 and EBI3 were markedly induced in PAs following TNFα stimulation. Kinetically, p28 was rapidly induced (<4 hrs), while peak induction of EBI3 was relatively delayed (>12 hrs) following exposure to TNFα. In contrast, only EBI3 was induced in ADs in response to TNFα, with magnitude and duration of mRNA expression similar to that of PAs. While mRNA expression of p28 was 'refractory' to TNFα stimulation in ADs, basal expression of p28 was significantly elevated (~60-fold) in ADs versus PAs (Fig.3.1A). To confirm that p28 was refractory to TNFα in ADs, PAs and ADs were stimulated in parallel with increasing concentrations of TNFα. Total RNA was harvested at 2 hrs post-TNFα stimulation and qRT-PCR used to assess p28 gene expression. As illustrated in Fig.3.1C, p28 mRNA expression increased in a concentration-dependent manner in PAs with an estimated ED50 of ~100pM, but remained unchanged in ADs even at TNFα concentrations as high as 1000pM.

Phenotypic differences between PAs and ADs regarding TNFα mediated changes in MAPK and NF-κB activity. Inflammatory activation of MAPK and NF-κB signaling pathways plays a central role in the pathogenesis of obesity-induced IR as well as IL-27 gene expression in immune-responsive cells (9,14,15,36-39). As data presented above demonstrate cell type specific regulation of p28 and EBI3 in response to TNFα (Fig.3.1A), we next assessed MAPK and NF-κB signaling in PAs and ADs in response to TNFα. To compare phenotypic differences, PAs and ADs were treated concurrently with TNFα and lysates harvested over time prior to immunoblotting with phosphospecific antibodies for JNK, ERK, and p38, where bisphosphorylation on both threonine and tyrosine residues is well documented as essential and sufficient for catalytic activity (40). Lysates were also immunoblotted for degradation of IκBα as an indirect measure of NF-κB nuclear translocation and activation (16). As shown in Fig.3.2, TNFα treatment resulted in transient activation of all three MAPKs in PAs, where robust phosphorylation was observed at 15 min with complete dephosphorylation by 60 min. TNFα stimulation also resulted in near complete IκBα degradation occurring with similar kinetics to that of MAPK phosphorylation in PAs. Conversely, the same concentration of TNFα produced markedly less magnitude, but sustained JNK and ERK phosphorylation with minimal activation of p38. While IκBα degradation occurred with similar magnitude and slight delay in ADs compared to PAs.

NF-κB regulates p28 and EBI3 in PAs in response to TNFα. To elucidate signaling events regulating p28 and EBI3 gene expression, PAs were pretreated with specific, pharmacological inhibitors of ERK (10 μM U0126), JNK (20 μM SP600125), p38 (10 μM SB203580), and NF-κB (10 μM Helenalin) for 30 min prior to 100 pM TNFα stimulation. Total RNA was harvested from PAs at 4hr and 18hr post-TNFα stimulation

corresponding to peak p28 and EBI3 expression. Changes in mRNA abundance were assessed by qRT-PCR and set relative to unstimulated controls. As illustrated in Fig.3.3, inhibition of NF-κB signaling markedly and selectively suppressed TNFα-induced p28 and EBI3 mRNA accumulation. As a measure of specificity, two other inflammatory genes, p19 and CCL2 were determined to be predominantly downstream of the MAPKs and not NF-κB using the same RNA preparation. Thus, these data clearly demonstrated that NF-κB represented a predominant signaling pathway regarding TNFα-induced changes in gene expression for both p28 and EBI3.

HDACs regulate TNFα-induced p28 gene expression, but not EBI3. Data presented above demonstrated that NF-κB signaling links TNFα action to p28 and EBI3 gene expression. However, as p28 and EBI3 expression was divergently regulated, these data further suggested a potential for epigenetics in the diverse outcomes observed with IL-27 regulation. Since acetylation has been linked to NF-κB activity in other reports, we investigated the impact of several broad-spectrum HDAC inhibitors on the regulation of p28 and EBI3 gene expression by TNFα. PAs were pretreated for 30 mins with trichostatin A (TSA; inhibitor of all HDACs except for the sirtuins), sodium butyrate (NaB; inhibitor of class I and II HDACs), or nicotinamide (NAM; inhibitor of sirtuin class III HDACs (41) prior to stimulation with 100 pM TNFα. As illustrated in Fig.3.4, all three HDAC inhibitors attenuated TNFα-induced p28 gene expression with the greatest effect shown with TSA. In contrast, TNFα-induced EBI3 mRNA accumulation was not suppressed by TSA, NaB, or NAM, demonstrating that HDAC activity was required for TNFα-induced p28, but not EBI3 gene expression.

Inhibitory effect of TSA on TNFα-induced p28 expression is independent of pretreatment time. As these genes were induced with markedly different kinetics, we next investigated a role for prolonged TSA treatment in the regulation of p28 and EBI3 gene expression. PAs were pretreated with TSA for 1 hr or 24 hr prior to total RNA collection at 2 hr and 12 hr post-TNFα stimulation. As illustrated in Fig.3.5A, TSA attenuated TNFα-induced expression of p28 independent of pretreatment time in PAs. In contrast, TSA did not suppress EBI3 expression at either pretreatment time confirming that TNFα-mediated p28 and EBI3 gene expression involved independent regulatory mechanisms. As p28 and EBI3 gene expression was divergently regulated based on cell phenotype, we also examined a role for HDACs on mRNA accumulation in ADs in the absence and presence of TNFα stimulation. ADs were pretreated with TSA for 1 hr or 24 hr and total RNA collected at 2hr and 12 hr post-TNFα. Consistent with results shown above, elevated p28 gene expression induced by differentiation was completely refractory to TNFα stimulation. Moreover, extended TSA pretreatment had no effect on differentiation induced p28 gene expression, nor the refractory nature of p28 to TNFα imposed by differentiation. While TNFα-induced EBI3 expression in ADs was not blocked by 1 hr TSA pretreatment, longer exposure to TSA increased TNFα-induced EBI3 expression, suggesting that HDACs may serve as co-repressors of NF-κB actions on EBI3 gene expression in ADs, but not PAs.

HDAC activity is not necessary for NF-κB signaling or nuclear translocation. As data presented above demonstrated that TSA suppressed TNFα induced p28 gene expression in PAs, we next examined the role of HDACs on TNFα induced changes in NF-κB signaling. PAs and ADs were pretreated with or without TSA for 1 hr and 24 hr and cell lysates collected 15 min post-TNFα stimulation. Lysates were

immunoblotted for IKKα/β phosphorylation as well as IκBα degradation as sequential events leading to NF-κB activation. As illustrated in Fig.3.6, TNFα increased IKKα/β phosphorylation as well as IκBα degradation in both PAs and ADs. Interestingly, TSA did not attenuate TNFα-induced IKKα/β phosphorylation or IκBα degradation suggesting that the regulatory role of HDACs regarding p28 gene expression occurred downstream of these initial events in NF-κB signaling. Therefore, we next examined the role of HDACs on NF-κB (p65) nuclear translocation. PAs and ADs were pretreated with or without TSA for 1 hr prior to TNFα stimulation. Nuclear and cytosolic extracts were harvested at 30 min post-TNFα and immunoblotted for NF-κB (p65). To confirm efficiency of fractionation, lysates were also immunoblotted for nucleoporin and α-tubulin as markers of nuclear and cytosolic fractions, respectively. As illustrated in Fig.3.7, TNFα induced nuclear NF-κB (p65) translocation was not blocked by TSA pretreatment suggesting that HDACs play a role in TNFα-induced p28 gene expression downstream of NF-κB translocation either through DNA interaction or by direct effects of histone acetylation important for p28, but not EBI3 gene expression.

Discussion

In this study, we present evidence for divergent regulation of IL-27 by HDACs in adipocytes. First, we show that the IL-27 subunits are differentially regulated in response to TNFα. While EBI3 was similarly induced in both PAs and ADs while p28 was only induced in PAs. Second, we demonstrate that EBI3 and p28 represent downstream targets of NF-κB signaling. Third, we report that the refractory nature of p28 in ADs was not due to differences in upstream NF-κB signaling, as this pathway was similarly

activated in PAs and ADs. Finally, we show that TNFα-induced p28 was attenuated with HDAC inhibition, potentially resulting from direct effects on chromatin acetylation. As external inflammatory cues are known to regulate gene expression through alterations of the epigenome, our data collectively support an epigenetic role for HDACs in the divergent regulation of p28, but not EBI3, in response to inflammatory stress in PAs, but not ADs.

Studies have established a significant role for IL-27 in the host defense against microbial infections, consequently leading to extensive examination into the regulation of IL-27 in immune cells. However, mechanisms that govern the regulation of IL-27 in non-immune cells have yet to be elucidated. Moreover, immune cell regulation of IL-27 has primarily focused on upstream activation by toll-like receptors (TLRs) (42,43). Data presented here demonstrate that both p28 and EBI3 are highly induced in adipocytes in response to TNFα. However, the kinetics of mRNA expression vary considerably between the two subunits suggesting that different mechanisms may be involved. Consistent with our data, others report that p28 and EBI3 kinetics were not coordinated within a given cell and that this differential mode of regulation was common among various cell types and tissues (43-45). These observations confirm that the individual subunits of IL-27 are differentially regulated in response to external cues, yet the detailed mechanisms for these divergent actions are still unclear.

Our data demonstrate that activation of NF-κB is the predominant signaling pathway that links TNFα to p28 and EBI3 gene expression in adipocytes. As TLR-mediated transcriptional regulation of p28 and EBI3 has been investigated in murine macrophages and dendritic cells (14,15), promoter analyses revealed the presence of

conserved NF-κB binding sites responsible for transcriptional activation. Furthermore, genetic ablation of NF-κB highlighted EBI3 as a downstream target of NF-κB activation (14). This observation is consistent with other studies suggesting that activation of NF-κB is required for EBI3 promoter activity, where mutations of NF-κB binding sites within the distal and proximal promoter markedly suppressed EBI3 activity (46,47). Similar to EBI3, NF-κB has been shown to enhance p28 promoter activity induced by LPS (15,36). Taken together, these data suggest that NF-κB binding is both necessary and sufficient for transcriptional activation of p28 and EBI3 expression in response to inflammatory stress.

Emerging evidence indicates that post-translational modification of NF-κB (e.g., acetylation, phosphorylation) (16,48,49) as well as recruitment of transcriptional co-activators and co-repressors is required for NF-κB transcriptional regulation of many inflammatory cytokines (18,23,50-52). As regulation of co-activators (e.g., HATs) and co-repressors (e.g., HDACs) is cell-type and condition-specific (53-56), post-translational modifications of the genome, or epigenetics, potentially provides one mechanism for the divergent biological outcomes observed with p28 and EBI3. Studies demonstrate that co-activator and co-repressor function is not universal, as these modulators of transcription can act in an opposing manner depending on cell-type and gene environments (57,58). HDACs are known co-repressors that function to deacetylate nucleosomal histones and alter chromatin in a manner that leads to gene repression. However, HDACs can deacetylate lysine residues on many proteins, including NF-κB (p65). Thus, HDACs can regulate NF-κB through deacetylation of regulatory elements within NF-κB-dependent inflammatory genes as well as post-translational modification of NF-κB proteins (59,60). Despite the general role for HDACs as transcriptional co-

repressors, it has been proposed that HDACs are necessary for gene expression (61). For example, deacetylation of p65 by HDACs has been shown to enhance NF-κB binding to the promoter region of select NF-κB target genes (62,63). Indeed, treatment with pan-HDAC inhibitors that blocks all HDACs have been shown to both enhance and suppress gene expression (64). Not surprisingly, HDAC inhibitors can promote NF-κB activity by increasing p65 acetylation (21,22,65) and diametrically act in an anti-inflammatory manner through the repression of inflammatory genes, demonstrating that HDAC activity is cell-type and environment specific (66-68). Consistent with this notion, we demonstrate that both p28 and EBI3 are downstream targets of NF-κB signaling and yet HDAC inhibition with TSA only ablated p28 gene expression. Taken together, these data suggest that divergent regulation of EBI3 and p28 gene expression may, in part, be under the control of epigenetic modifications of nucleosomal chromatin and not upstream NF-κB signaling.

Recent evidence further highlights HDACs in the regulation of adipogenesis (69-71). Chatterjee et al. demonstrated that class II HDAC9, is significantly down-regulated during adipocyte differentiation. Gain- and loss-of-function studies further demonstrated that HDAC9 acts as a molecular switch to regulate adipogenic gene expression and differentiation (69). Moreover, reports have shown that treatment with pan-HDAC inhibitors block adipogenesis (72). Our findings demonstrated that p28 gene expression was elevated with adipocyte differentiation, while EBI3 was suppressed. In addition, p28 expression was not induced with TNFα in ADs, nor suppressed in the presence of HDAC inhibition. However, TNFα-induced EBI3 gene expression was further exacerbated with HDAC inhibition. Collectively, these data suggest that HDAC activity is necessary for TNFα-induced p28 gene expression in PAs, but not required in ADs. Conversely, HDAC

activity represses EBI3 expression during adipocyte differentiation and loss of HDAC repression enhances EBI3 gene expression in response to inflammation. Thus, epigenetic alterations of the *IL-27 gene* may explain the divergent outcomes observed in PAs and ADs.

In summary, this study provides convincing evidence for the epigenetic regulation of IL-27 under conditions of inflammatory stress and in response to adipocyte differentiation. Data presented here demonstrate that two subunits of IL-27 (p28 and EBI3) are differentially regulated in adipocytes in response to TNFα, even though both p28 and EBI3 were downstream of NF-κB. Thus, it appears that external inflammatory stress alters the network of chemical switches, in particular acetylation, within cells collectively known as the epigenome that allows for NF-κB binding and gene expression. Conclusions drawn from our data have led to the following model whereby HDACs regulate p28 gene expression in PAs but not ADs (Fig.3.8). In contrast, regulation of EBI3 from TNFα is independent of HDAC activity and cellular differentiation (Fig.3.8). These data highlight the potential role for epigenetics in IL-27 regulation and may lead to novel epigenetic therapies combating obesity-induced inflammation, IR and ensuing diabetes.

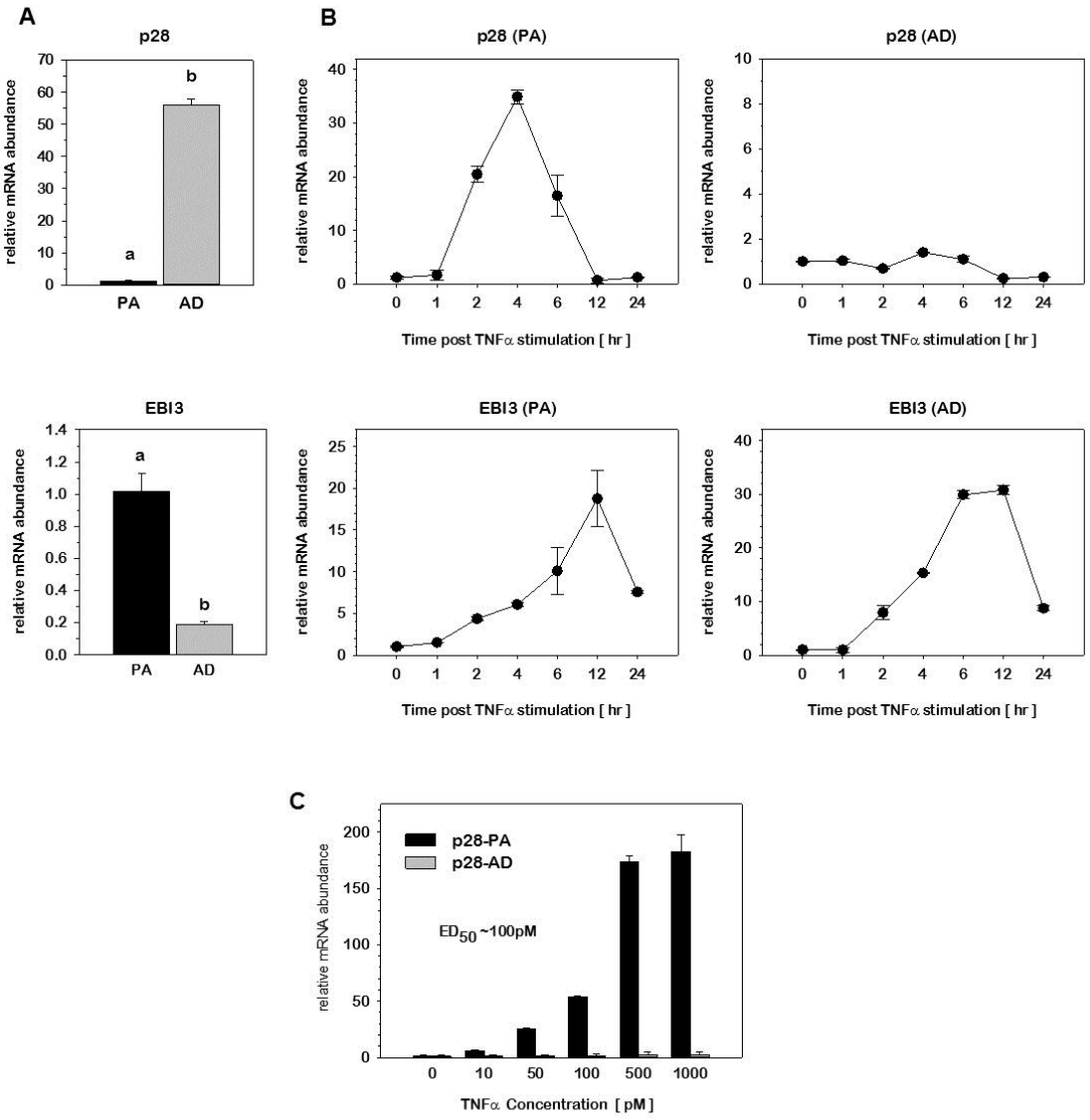

Figure 3.1. Phenotype difference in TNFα-induced IL-27 expression in PAs and ADs. (A) Relative mRNA abundance for p28 and EBI3 of PAs and ADs was measured using qRT-PCR. Data were normalized to 18S rRNA and expressed relative to PAs. Statistical significance was determined by Student's t-test (* $p<0.05$). (B) PAs and ADs were stimulated in parallel with 100 pM TNFα and changes in mRNA abundance determined over time relative to unstimulated (0 hr) conditions. (C) PAs and ADs were stimulated in parallel with increasing concentrations of TNFα. Total RNA harvested at 2 hr post-TNFα stimulation and p28 mRNA abundance was determined relative to unstimulated cells (0 pM).

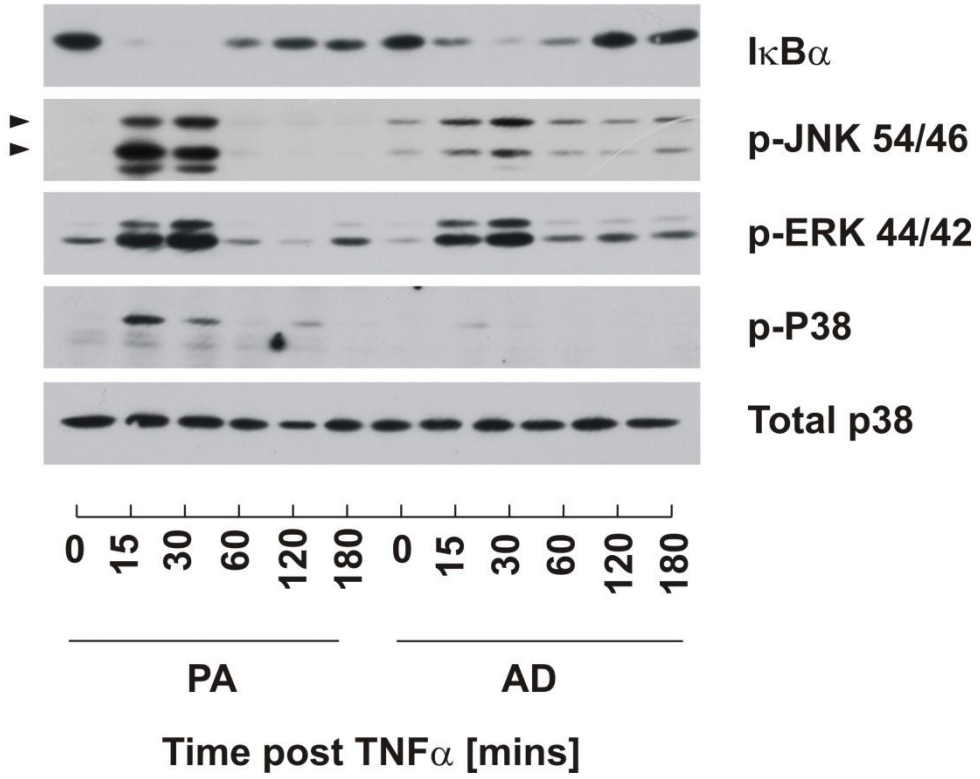

Figure 3.2. Phenotypic differences in TNFα-stimulated MAPK and NF-κB signaling between PAs and ADs. Cell lysates were harvested from PAs and ADs over time following TNFα (100 pM) stimulation and immunoblotted for p-ERK, p-JNK, p-p38 as well as IκBα degradation. Total p38 was included as a loading control.

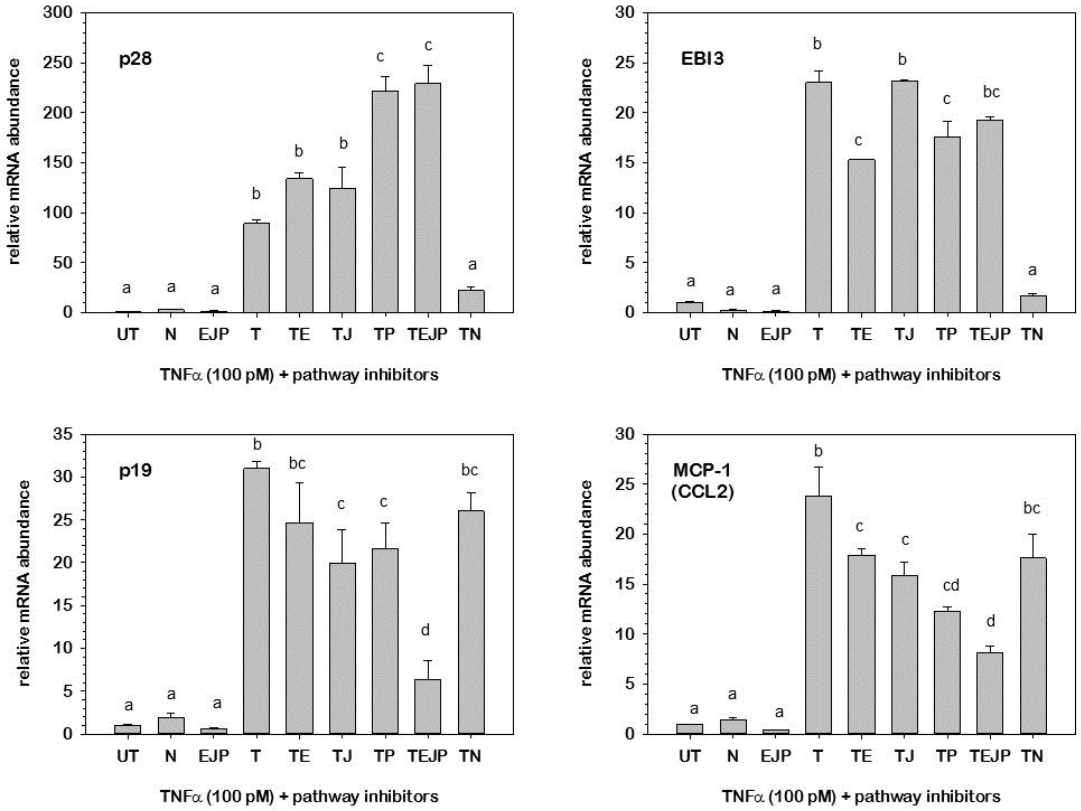

Figure 3.3. NF-κB signaling regulates TNFα-induced p28 and EBI3 gene expression. PAs were pretreated for 1 hr with inhibitors for ERK (U0126, 10 μM), JNK (SP600125, 20 μM), p38 (SB203850, 10 μM), or NF-κB (Helenalin, 30 μM) prior to TNFα stimulation. Total RNA was harvested at 2 hr or 18 hr post-TNFα and mRNA expression analyzed for p28, EBI3, p19, and CCL2. Data were normalized to 18S rRNA and expressed relative to untreated (UT) controls. Statistical significance was determined by ANOVA with Tukey's *post-hoc* analysis conducted to assess differences between treatment groups when $p<0.05$. Abbreviations used: TNFα (T), ERK (E), JNK (J), p38 (P), and NF-κB (N).

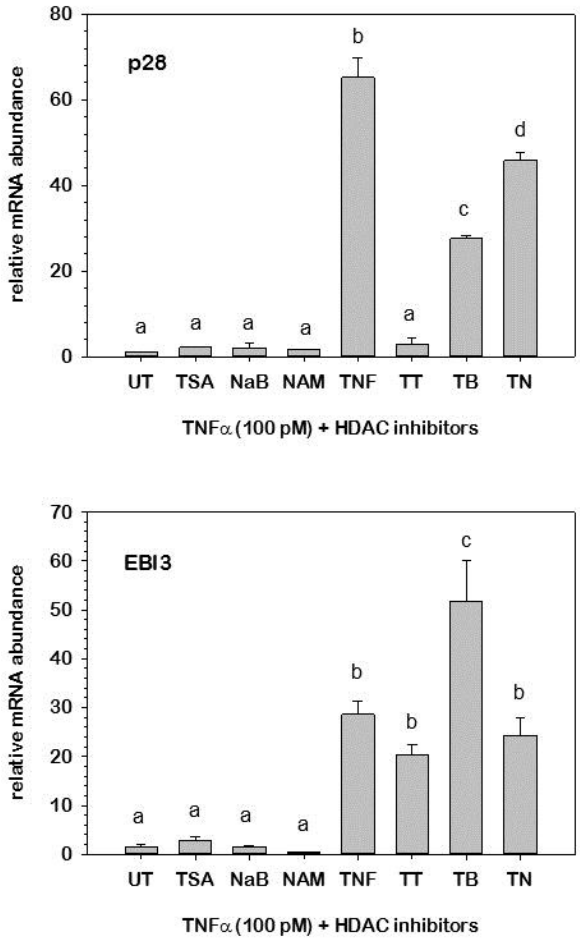

Figure 3.4. Effect of HDAC inhibitors on TNFα-induced p28 and EBI3 gene expression. PAs were pretreated for 1 hr with HDAC inhibitors, TSA (0.5 μM), NaB (1 mM), or NAM (5 mM) prior to 100 pM TNFα stimulation. Total RNA was harvested at 2 hr and 12 hr post-TNFα stimulation and assessed for p28 and EBI3 mRNA expression via qRT-PCR, respectively. Data were normalized to 18S rRNA and expressed related to untreated (UT) controls. Statistical significance was determined by ANOVA with Tukey's *post-hoc* analysis conducted to assess differences between treatment groups when $p<0.05$.

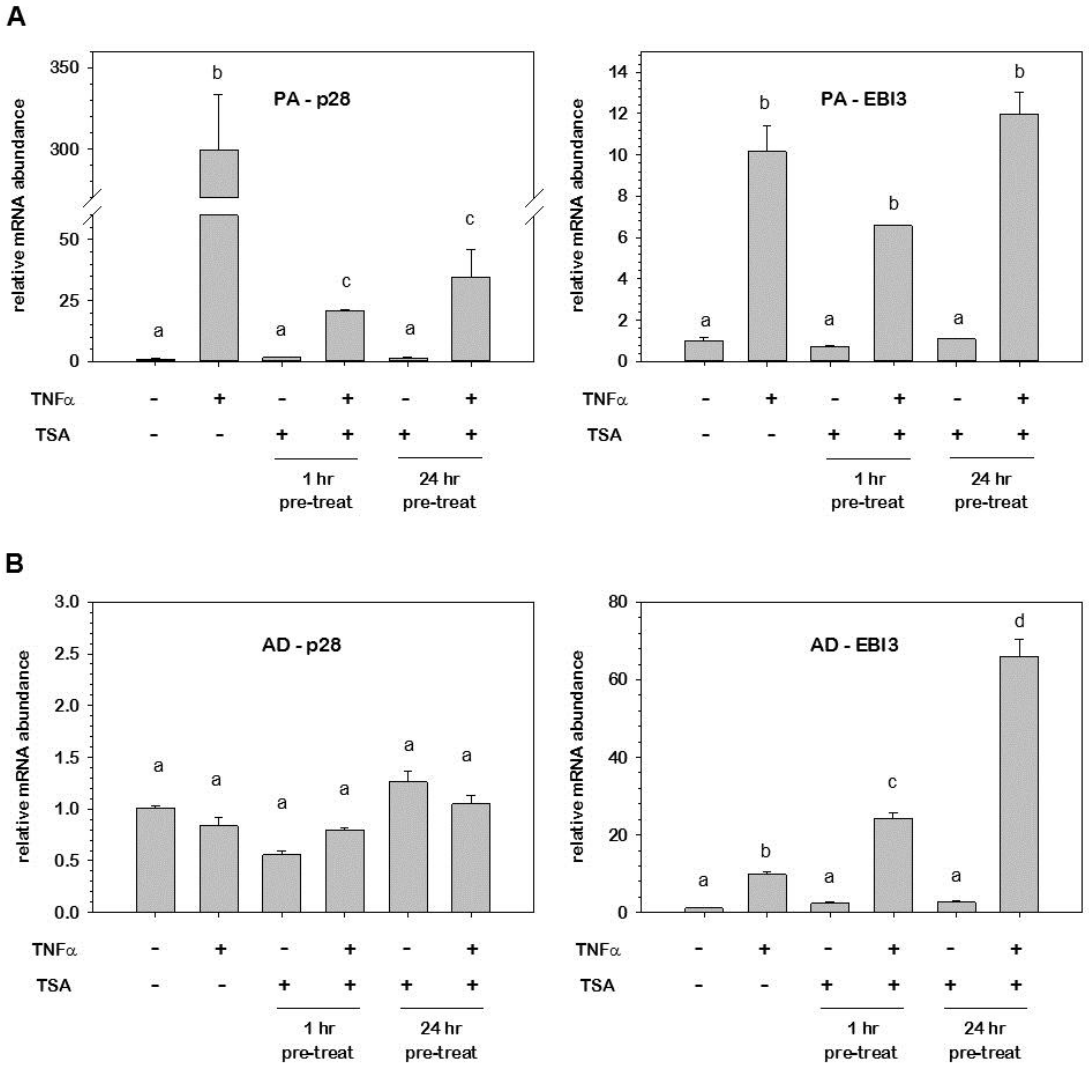

Figure 3.5. Effects of TSA on TNFα-induced p28 and EBI3 gene expression are independent with exposure time. PAs (A) and ADs (B) were pretreated for 1 hr or 24 hr in the presence of TSA (0.5 μM) prior to 100 pM TNFα stimulation. Total RNA was harvested at 2 hr or 12 hr post-TNFα stimulation and assessed for p28 and EBI3 expression via qRT-PCR, respectively. Data were normalized to 18S rRNA and expressed relative to untreated controls. Statistical significance was determined by ANOVA with Tukey's *post-hoc* analysis conducted to assess differences between treatment groups when $p<0.05$.

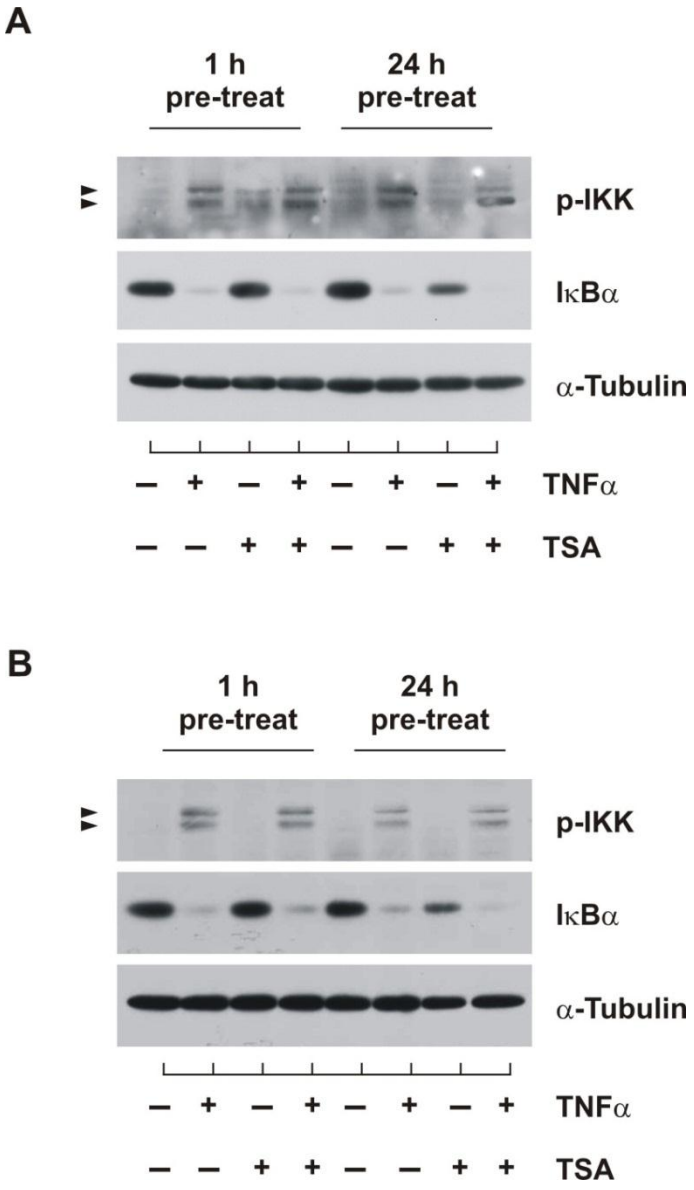

Figure 3.6. TSA does not inhibit TNFα-induced NF-κB signaling pathway activation. PAs (A) and ADs (B) were pretreated for 1 hr or 24 hr in the presence of TSA (0.5 μM) prior to 100 pM TNFα stimulation. Cell lysates were harvested at 15 min post-TNFα stimulation and immunoblotted for p-IKK and IκBα degradation. α-tubulin was included as a loading control.

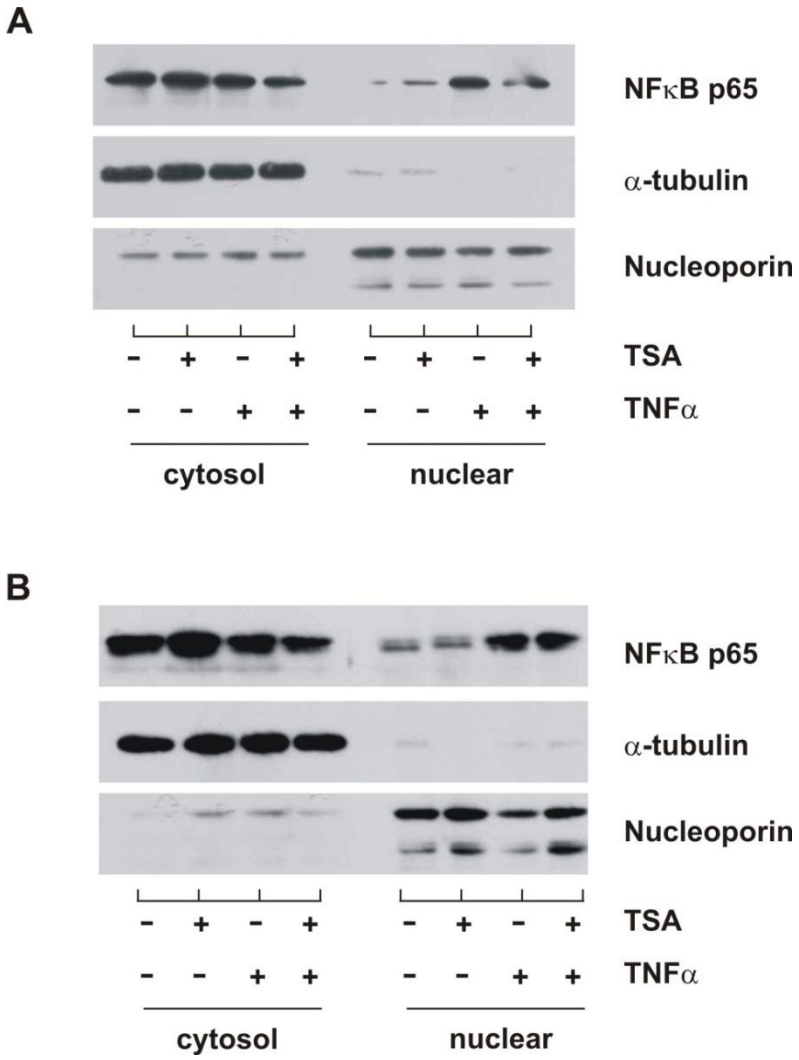

Figure 3.7. TSA does not block TNFα-induced NF-κB nuclear translocation. Nuclear and cytosolic extracts were harvested from PAs (A) and ADs (B) at 30 min after TNFα stimulation in the absence and presence of TSA (0.5 μM) and immunoblotted for NF-κB(p65). α-tubulin and nucleoporin were used as markers of cytosolic and nuclear fractions, respectively.

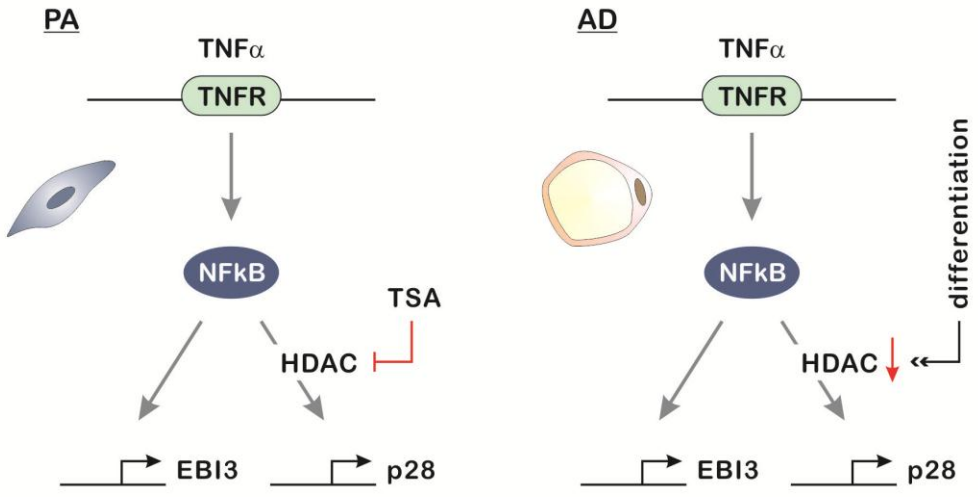

Figure 3.8. Working model for regulation of p28 and EBI3 gene expression by HDACs. In PAs, EBI3 gene is expressed in response to TNFα directly through NF-κB signaling events independent of HDAC activity. In contrast, TNFα induced p28 gene expression is postulated to require specific HDAC activity that decreases during adipocyte differentiation resulting in the refractory nature of p28 regarding inflammatory stress in ADs.

Reference List

1. Guilherme A, Virbasius JV, Puri V, Czech MP. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. Nat Rev Mol Cell Biol. 2008 May;9:367-77.

2. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest. 2005 May;115:1111-9.

3. Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. J Clin Invest. 2003 Dec;112:1785-8.

4. Hotamisligil GS, Erbay E. Nutrient sensing and inflammation in metabolic diseases. Nat Rev Immunol. 2008 Dec;8:923-34.

5. Kanda H, Tateya S, Tamori Y, Kotani K, Hiasa K, Kitazawa R, Kitazawa S, Miyachi H, Maeda S, et al. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. J Clin Invest. 2006 Jun;116:1494-505.

6. Pflanz S, Timans JC, Cheung J, Rosales R, Kanzler H, Gilbert J, Hibbert L, Churakova T, Travis M, et al. IL-27, a heterodimeric cytokine composed of EBI3 and p28 protein, induces proliferation of naive CD4(+) T cells. Immunity. 2002 Jun;16:779-90.

7. Hunter CA. New IL-12-family members: IL-23 and IL-27, cytokines with divergent functions. Nat Rev Immunol. 2005 Jul;5:521-31.

8. Coulomb-L'Hermine A, Larousserie F, Pflanz S, Bardel E, Kastelein RA, Devergne O. Expression of interleukin-27 by human trophoblast cells. Placenta. 2007 Nov;28:1133-40.

9. Kempe S, Heinz P, Kokai E, Devergne O, Marx N, Wirth T. Epstein-barr virus-induced gene-3 is expressed in human atheroma plaques. Am J Pathol. 2009 Jul;175:440-7.

10. Kempe S, Kestler H, Lasar A, Wirth T. NF-kappaB controls the global pro-inflammatory response in endothelial cells: evidence for the regulation of a pro-atherogenic program. Nucleic Acids Res. 2005;33:5308-19.

11. Febbraio MA. gp130 receptor ligands as potential therapeutic targets for obesity. J Clin Invest. 2007 Apr;117:841-9.

12. White UA, Stewart WC, Mynatt RL, Stephens JM. Neuropoietin attenuates adipogenesis and induces insulin resistance in adipocytes. J Biol Chem. 2008 Aug 15;283:22505-12.

13. Song H, Lim H. Evidence for heterodimeric association of leukemia inhibitory factor (LIF) receptor and gp130 in the mouse uterus for LIF signaling during blastocyst implantation. Reproduction. 2006 Feb;131:341-9.

14. Wirtz S, Becker C, Fantini MC, Nieuwenhuis EE, Tubbe I, Galle PR, Schild HJ, Birkenbach M, Blumberg RS, Neurath MF. EBV-induced gene 3 transcription is induced by TLR signaling in primary dendritic cells via NF-kappa B activation. J Immunol. 2005 Mar 1;174:2814-24.

15. Liu J, Guan X, Ma X. Regulation of IL-27 p28 gene expression in macrophages through MyD88- and interferon-gamma-mediated pathways. J Exp Med. 2007 Jan 22;204:141-52.

16. Perkins ND. Integrating cell-signalling pathways with NF-kappaB and IKK function. Nat Rev Mol Cell Biol. 2007 Jan;8:49-62.

17. Wan F, Lenardo MJ. The nuclear signaling of NF-kappaB: current knowledge, new insights, and future perspectives. Cell Res. 2010 Jan;20:24-33.

18. Hayden MS, Ghosh S. Shared principles in NF-kappaB signaling. Cell. 2008 Feb 8;132:344-62.

19. Ghosh S, Hayden MS. New regulators of NF-kappaB in inflammation. Nat Rev Immunol. 2008 Nov;8:837-48.

20. Chen CJ, Deng Z, Kim AY, Blobel GA, Lieberman PM. Stimulation of CREB binding protein nucleosomal histone acetyltransferase activity by a class of transcriptional activators. Mol Cell Biol. 2001 Jan;21:476-87.

21. Quivy V, Van LC. Regulation at multiple levels of NF-kappaB-mediated transactivation by protein acetylation. Biochem Pharmacol. 2004 Sep 15;68:1221-9.

22. Liu Y, Xia X, Fondell JD, Yen PM. Thyroid hormone-regulated target genes have distinct patterns of coactivator recruitment and histone acetylation. Mol Endocrinol. 2006 Mar;20:483-90.

23. Gao Z, Chiao P, Zhang X, Zhang X, Lazar MA, Seto E, Young HA, Ye J. Coactivators and corepressors of NF-kappaB in IkappaB alpha gene promoter. J Biol Chem. 2005 Jun 3;280:21091-8.

24. Yeung F, Hoberg JE, Ramsey CS, Keller MD, Jones DR, Frye RA, Mayo MW. Modulation of NF-kappaB-dependent transcription and cell survival by the SIRT1 deacetylase. EMBO J. 2004 Jun 16;23:2369-80.

25. Yin L, Laevsky G, Giardina C. Butyrate suppression of colonocyte NF-kappa B activation and cellular proteasome activity. J Biol Chem. 2001 Nov 30;276:44641-6.

26. Hu J, Colburn NH. Histone deacetylase inhibition down-regulates cyclin D1 transcription by inhibiting nuclear factor-kappaB/p65 DNA binding. Mol Cancer Res. 2005 Feb;3:100-9.

27. Place RF, Noonan EJ, Giardina C. HDAC inhibition prevents NF-kappa B activation by suppressing proteasome activity: down-regulation of proteasome subunit expression stabilizes I kappa B alpha. Biochem Pharmacol. 2005 Aug 1;70:394-406.

28. Furumai R, Ito A, Ogawa K, Maeda S, Saito A, Nishino N, Horinouchi S, Yoshida M. Histone deacetylase inhibitors block nuclear factor-kappaB-dependent transcription by interfering with RNA polymerase II recruitment. Cancer Sci. 2011 May;102:1081-7.

29. Wolter S, Doerrie A, Weber A, Schneider H, Hoffmann E, von der OJ, Bakiri L, Wagner EF, Resch K, Kracht M. c-Jun controls histone modifications, NF-kappaB recruitment, and RNA polymerase II function to activate the ccl2 gene. Mol Cell Biol. 2008 Jul;28:4407-23.

30. Djian P, Phillips M, Green H. The activation of specific gene transcription in the adipose conversion of 3T3 cells. J Cell Physiol. 1985 Sep;124:554-6.

31. Morrison RF, Farmer SR. Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. J Biol Chem. 1999 Jun 11;274:17088-97.

32. Ferguson BS, Nam H, Hopkins RG, Morrison RF. Impact of reference gene selection for target gene normalization on experimental outcome using real-time qRT-PCR in adipocytes. PLoS One. 2010;5:e15208.

33. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25:402-8.

34. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993 Jan 1;259:87-91.

35. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature. 1997 Oct 9;389:610-4.

36. Zhang J, Qian X, Ning H, Yang J, Xiong H, Liu J. Activation of IL-27 p28 gene transcription by interferon regulatory factor 8 in cooperation with interferon regulatory factor 1. J Biol Chem. 2010 Jul 9;285:21269-81.

37. Marriott JB, Clarke IA, Dalgleish AG. Inhibition of p38 MAP kinase during cellular activation results in IFN-gamma-dependent augmentation of IL-12 production by human monocytes/macrophages. Clin Exp Immunol. 2001 Jul;125:64-70.

38. Zhang S, Kaplan MH. The p38 mitogen-activated protein kinase is required for IL-12-induced IFN-gamma expression. J Immunol. 2000 Aug 1;165:1374-80.

39. Zhang JS, Feng WG, Li CL, Wang XY, Chang ZL. NF-kappa B regulates the LPS-induced expression of interleukin 12 p40 in murine peritoneal macrophages: roles of PKC, PKA, ERK, p38 MAPK, and proteasome. Cell Immunol. 2000 Aug 25;204:38-45.

40. Pearson G, Robinson F, Beers GT, Xu BE, Karandikar M, Berman K, Cobb MH. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev. 2001 Apr;22:153-83.

41. McKinsey TA. Isoform-selective HDAC inhibitors: closing in on translational medicine for the heart. J Mol Cell Cardiol. 2011 Oct;51:491-6.

42. Heidemann J, Ruther C, Kebschull M, Domschke W, Bruwer M, Koch S, Kucharzik T, Maaser C. Expression of IL-12-related molecules in human intestinal microvascular endothelial cells is regulated by TLR3. Am J Physiol Gastrointest Liver Physiol. 2007 Dec;293:G1315-G1324.

43. Pirhonen J, Siren J, Julkunen I, Matikainen S. IFN-alpha regulates Toll-like receptor-mediated IL-27 gene expression in human macrophages. J Leukoc Biol. 2007 Nov;82:1185-92.

44. Sonobe Y, Yawata I, Kawanokuchi J, Takeuchi H, Mizuno T, Suzumura A. Production of IL-27 and other IL-12 family cytokines by microglia and their subpopulations. Brain Res. 2005 Apr 8;1040:202-7.

45. Schuetze N, Schoeneberger S, Mueller U, Freudenberg MA, Alber G, Straubinger RK. IL-12 family members: differential kinetics of their TLR4-mediated induction by Salmonella enteritidis and the impact of IL-10 in bone marrow-derived macrophages. Int Immunol. 2005 May;17:649-59.

46. Bell S, Degitz K, Quirling M, Jilg N, Page S, Brand K. Involvement of NF-kappaB signalling in skin physiology and disease. Cell Signal. 2003 Jan;15:1-7.

47. O'Neil J, Ventura JJ, Cusson N, Kelliher M. NF-kappaB activation in premalignant mouse tal-1/scl thymocytes and tumors. Blood. 2003 Oct 1;102:2593-6.

48. Huang B, Yang XD, Lamb A, Chen LF. Posttranslational modifications of NF-kappaB: another layer of regulation for NF-kappaB signaling pathway. Cell Signal. 2010 Sep;22:1282-90.

49. Campbell KJ, Perkins ND. Post-translational modification of RelA(p65) NF-kappaB. Biochem Soc Trans. 2004 Dec;32:1087-9.

50. Baek SH, Ohgi KA, Rose DW, Koo EH, Glass CK, Rosenfeld MG. Exchange of N-CoR corepressor and Tip60 coactivator complexes links gene expression by NF-kappaB and beta-amyloid precursor protein. Cell. 2002 Jul 12;110:55-67.

51. Sheppard KA, Rose DW, Haque ZK, Kurokawa R, McInerney E, Westin S, Thanos D, Rosenfeld MG, Glass CK, Collins T. Transcriptional activation by NF-kappaB requires multiple coactivators. Mol Cell Biol. 1999 Sep;19:6367-78.

52. Ashburner BP, Westerheide SD, Baldwin AS, Jr. The p65 (RelA) subunit of NF-kappaB interacts with the histone deacetylase (HDAC) corepressors HDAC1 and HDAC2 to negatively regulate gene expression. Mol Cell Biol. 2001 Oct;21:7065-77.

53. Francis GA, Fayard E, Picard F, Auwerx J. Nuclear receptors and the control of metabolism. Annu Rev Physiol. 2003;65:261-311.

54. Christian M, Kiskinis E, Debevec D, Leonardsson G, White R, Parker MG. RIP140-targeted repression of gene expression in adipocytes. Mol Cell Biol. 2005 Nov;25:9383-91.

55. Fritah A. Control of skeletal muscle metabolic properties by the nuclear receptor corepressor RIP140. Appl Physiol Nutr Metab. 2009 Jun;34:362-7.

56. Zschiedrich I, Hardeland U, Krones-Herzig A, Berriel DM, Vegiopoulos A, Muggenburg J, Sombroek D, Hofmann TG, Zawatzky R, et al. Coactivator function of RIP140 for NFkappaB/RelA-dependent cytokine gene expression. Blood. 2008 Jul 15;112:264-76.

57. Rogatsky I, Zarember KA, Yamamoto KR. Factor recruitment and TIF2/GRIP1 corepressor activity at a collagenase-3 response element that mediates regulation by phorbol esters and hormones. EMBO J. 2001 Nov 1;20:6071-83.

58. Jepsen K, Hermanson O, Onami TM, Gleiberman AS, Lunyak V, McEvilly RJ, Kurokawa R, Kumar V, Liu F, et al. Combinatorial roles of the nuclear receptor corepressor in transcription and development. Cell. 2000 Sep 15;102:753-63.

59. Yang XJ, Seto E. Lysine acetylation: codified crosstalk with other posttranslational modifications. Mol Cell. 2008 Aug 22;31:449-61.

60. Minucci S, Pelicci PG. Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer. Nat Rev Cancer. 2006 Jan;6:38-51.

61. Smith JJ, Torigoe SE, Maxson J, Fish LC, Wiley EA. A class II histone deacetylase acts on newly synthesized histones in Tetrahymena. Eukaryot Cell. 2008 Mar;7:471-82.

62. Chen LF, Greene WC. Shaping the nuclear action of NF-kappaB. Nat Rev Mol Cell Biol. 2004 May;5:392-401.

63. Kiernan R, Bres V, Ng RW, Coudart MP, El MS, Sardet C, Jin DY, Emiliani S, Benkirane M. Post-activation turn-off of NF-kappa B-dependent transcription is regulated by acetylation of p65. J Biol Chem. 2003 Jan 24;278:2758-66.

64. Halili MA, Andrews MR, Labzin LI, Schroder K, Matthias G, Cao C, Lovelace E, Reid RC, Le GT, et al. Differential effects of selective HDAC inhibitors on macrophage inflammatory responses to the Toll-like receptor 4 agonist LPS. J Leukoc Biol. 2010 Jun;87:1103-14.

65. Chen L, Fischle W, Verdin E, Greene WC. Duration of nuclear NF-kappaB action regulated by reversible acetylation. Science. 2001 Aug 31;293:1653-7.

66. Blanchard F, Chipoy C. Histone deacetylase inhibitors: new drugs for the treatment of inflammatory diseases? Drug Discov Today. 2005 Feb 1;10:197-204.

67. Adcock IM. HDAC inhibitors as anti-inflammatory agents. Br J Pharmacol. 2007 Apr;150:829-31.

68. Calao M, Burny A, Quivy V, Dekoninck A, Van LC. A pervasive role of histone acetyltransferases and deacetylases in an NF-kappaB-signaling code. Trends Biochem Sci. 2008 Jul;33:339-49.

69. Chatterjee TK, Idelman G, Blanco V, Blomkalns AL, Piegore MG, Jr., Weintraub DS, Kumar S, Rajsheker S, Manka D, et al. Histone deacetylase 9 is a negative regulator of adipogenic differentiation. J Biol Chem. 2011 Aug 5;286:27836-47.

70. Yoo EJ, Chung JJ, Choe SS, Kim KH, Kim JB. Down-regulation of histone deacetylases stimulates adipocyte differentiation. J Biol Chem. 2006 Mar 10;281:6608-15.

71. Kim SN, Choi HY, Kim YK. Regulation of adipocyte differentiation by histone deacetylase inhibitors. Arch Pharm Res. 2009 Apr;32:535-41.

72. Haberland M, Carrer M, Mokalled MH, Montgomery RL, Olson EN. Redundant control of adipogenesis by histone deacetylases 1 and 2. J Biol Chem. 2010 May 7;285:14663-70.

CHAPTER IV

NOVEL MECHANISMS OF GOLGI-MEDIATED TNFα-INDUCED INFLAMMATORY GENE EXPRESSION IN ADIPOCYTES

Abstract

Tumor necrosis factor (TNF) α is pleiotropic cytokine that links obesity and IR. TNFα binds cell surface receptors (TNFR1/2) on adipocytes subsequently activating inflammatory signaling cascades that alter adipocyte function and exacerbate the inflammatory response. Evidence suggests that the Golgi apparatus (GA) plays a crucial role in TNFα signaling through redistribution of TNFα receptors (TNFRs). Despite these implications, no study has addressed a role for the GA on TNFα action in adipocytes. To address this question, we stimulated 3T3-L1 murine adipocytes with TNFα in the presence of a well-characterized pharmacological GA inhibitor, brefeldin A (BFA). We report that BFA significantly ablated TNFα-induced inflammatory gene expression; effects did not occur in the presence of lipopolysaccharide (LPS) or interferon (IFN) γ suggesting that the anti-inflammatory actions of BFA were specific to TNFα. We further demonstrate that BFA blocked TNFR1 and TNFR2 cell-surface expression, suggesting that a regulatory role for the GA on TNFα signaling in the cell. While pre-treatment with BFA blocked TNFα-induced MAPK and NF-κB signaling in a manner consistent with the effects on gene expression, co-treatment with BFA ablated inflammatory gene expression with no measurable attenuation of MAPKs or NF-κB signaling. Collectively, these findings demonstrate that the GA is a critical regulator of TNFα-induced

inflammation in adipocytes and highlights a novel target for the potential treatment of obesity-associated AT inflammation.

Introduction

Chronic inflammation plays a central role in coupling obesity and insulin resistance (1-3). Mounting evidence demonstrates that increased adiposity, associated with obesity, often results in elevated cytokine and chemokine secretion from adipocytes leading to AT remodeling by infiltrating macrophages and other cells of the immune system (4). The result is an exacerbated inflammatory response and the development of local and systemic IR. Over two decades of evidence has clearly positioned, TNFα, front and foremost, as a significant contributor to this pathophysiological association. This pleiotropic cytokine, which is elevated in animal and human AT tissue during development of genetic and diet-induced obesity, is well documented to reduce insulin sensitivity in vivo and in vitro (5,6). Furthermore, TNFα ablation, by immunosorbent strategies or targeted gene deletion, dramatically reduces ensuing insulin resistance (7). As mediating mechanisms impinge on insulin signaling pathways in the cytosol as well as inflammatory gene expression in the nucleus, it is not unexpected that numerous processes have evolved to mitigate TNFα action under non-inflammatory conditions.

TNFα action is transduced in adipocytes through two cell surface receptors known as TNFR1 (p55) and TNFR2 (p75) (8). Although TNFα can bind and signal through TNFR2, most of its activity regarding inflammatory gene expression and IR occurs through TNFR1 where ligand binding leads to internalization of the ligand-receptor complex (9,10). While some TNFα mediated-events depend on internalization,

others are terminated as the receptor is recycled through recycling endosomes back to the membrane with degradation of the ligand by lysosomal activity. If not recycled or replaced, the outcome of internalization would be a decrease in surface receptor density leading to a decrease in TNFα sensitivity. A similar loss in TNFR on the surface occurs following enzymatic cleavage of membrane-bounded receptors by TACE resulting in truncated, non-functional, soluble receptors (i.e., sTNFR) that are known to play a role in dampening TNFα activity (11,12). Thus, vesicular trafficking of TNFR, either from the de novo synthesis in the endoplasmic reticulum (ER) or through recycling endosome is critical for TNFα action. To this point, others have also reported that TNFR1 is predominantly localized to Golgi apparatus (GA) in a perinuclear compartment as opposed to cell surface expression providing a means to curtail receptor sensitivity when TNFα action is not warranted. As this pool of TNFR1 has been shown to translocate to the surface upon TNFα stimulation, it has also been suggested that the Golgi pool serves a reservoir to increase cell surface receptor density when TNFα action is warranted (13). It has also been demonstrated that the cytoplasmic domain of the TNFR1 possess a Golgi-localization sequence (14,15), but how and under what conditions this localized trafficking occurs has yet to be determined. Furthermore, to date, Golgi-associated TNFR1 has been reported for only a few cell types suggesting the possibility of a cell-type, condition-specific phenomenon.

As studies have clearly demonstrated the dynamic nature of TNFR1 surface expression influenced by receptor internalization as well as receptor shedding, vesicular trafficking, which may or may not involve Golgi activity, represents an important regulatory mechanism mediating TNFα action. Despite the critical importance of TNFα signaling in the etiology of obesity-induced IR, no information regarding a role for the GA

in regulating TNFα action in adipocytes has emerged. In this study, we present evidence demonstrating obligatory roles for the GA in mediating TNFα induced inflammatory gene expression in the adipocytes. Data are presented demonstrating a role for the GA in maintaining TNFR surface density as well as an unreported role for this perinuclear organelle on inflammatory gene expression that is independent of surface density or signaling pathways that mediate TNFα action.

Materials and Methods

Materials. Dulbecco's Modified Eagle's Medium (DMEM), calf bovine serum (CS), Trypsin-EDTA, and recombinant murine TNFα were purchased from Invitrogen. Fetal bovine serum (FBS) was obtained from HyClone. The following antibodies were used for western blot analysis: Phospho-ERK (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-JNK (Thr183/Tyr185), phospho-ATF2 (Thr69/71), phosphor-c-JUN (Ser63), IκBα, and α-tubulin (Cell Signaling). Brefeldin A (BFA), monensin (MNS), cycloheximide (CHX), and lipopolysaccharide (LPS) were obtained from Sigma. Mouse recombinant interferon (IFN) γ, mouse polyclonal anti-goat TNFR1, TNFR2, control IgG, and donkey anti-goat IgG (H+L) phycoerythrin-conjugated antibodies were obtained from R&D systems. Enhanced chemiluminescence (ECL) reagents were obtained from Perkin-Elmer Life Sciences. All TaqMan primer probes used in this study were purchased from Applied Biosystems.

Cell culture. The murine 3T3-L1 cell line was purchased from Howard Green, Harvard Medical School (16). Cells were propagated in DMEM supplemented with 10% CS until density-induced arrest, as previously described (17). At 2 days post-confluence,

growth medium was replaced with DMEM supplemented with 10% FBS, 0.5 mM 1-methy-3-isobutylxanthane, 1 µM dexamethasone, and 1.7 µM insulin (MDI) for 2 days. Subsequently, cells were cultured in DMEM supplemented only with 10% FBS over the following six days as preadipocytes (PAs) differentiated into mature adipocytes (ADs). Throughout the study, 'time 0' refers to density arrested cells immediately before the addition of MDI to the culture medium. Experiments described herein were conducted in density-arrested PAs (d0) or mature ADs (d8) pretreated with 1 µM BFA, 30 µM MNS, or 5 µM CHX in indicated time points then stimulated with 100 pM TNFα, 100 ng/ml LPS, or 20 ng/ml IFNγ. All experiments were repeated 2-3 times to validate results and ensure reliability.

Immunoblotting. Cell monolayers were washed with phosphate-buffer saline (PBS) and scraped into ice-cold lysis buffer containing 0.1 M Tris (pH 7.4), 150 mM NaCl, 10% sodium dodecyl sulfate (SDS), 1% Triton X, 0.5% Nonidet P-40 (NP40), 1 mM EDTA, 1 mM EGTA. Phosphatase inhibitors (20 mM β-glycerophosphate, 10 mM sodium fluoride and 2 µM sodium orthovanadate) and protease inhibitors (0.3 µM aprotinin, 21 µM leupetin, 1 µM pepstatin, 50 µM phenanthroline, 0.5 µM phenylmethylsulfonyl fluoride) were added to lysis buffer immediately prior to cell harvest. Cell lysates were sonicated and centrifuged (13,000*g,* 10 min, 4ºC), and the supernatant transferred to a fresh tube. Protein content was determined by bicinchoninic acid (BCA) procedures according to manufacturer's (Pierce, Rockford, IL) instructions. Equal amounts of whole cell lysate protein were separated by SDS-PAGE electrophoresis. Cell lysates were mixed with loading buffer containing 0.25 M Tris (pH 6.8), 4% SDS, 10% glycerol, 0.01% bromophenol blue, and 10% dithiothreitol, then heated at 80ºC for 5 min prior to electrophoresis. Proteins were resolved on SDS-

polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore corp., Billerica, MA). After transfer, membranes were blocked with 4% milk and probed with indicated primary antibodies overnight at 4ºC. Membranes were subsequently probed with horseradish peroxidase-conjugated secondary antibodies for 1h at room temperature. Membranes were immersed in ECL and data visualized by autoradiography using CL-XPosure film (Pierce).

Real-time qRT-PCR. Total RNA was extracted and genomic DNA contamination was removed using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA), according to manufacturer protocol. Total RNA was quantified with a Nanodrop ND-1000 spectrophotometer and reverse transcribed to cDNA in a 10 µl reaction volume using a high capacity cDNA reverse transcription kit (Applied Biosystems). The reverse transcription (RT) master mix containing RT buffer, deoxyribonucleotide triphosphate (dNTP) mix, RT random primers, RNase inhibitor (1.0 U/µl), and MultiScribe RT was added to 1 µg RNA and RNase-free water. Reverse transcription reaction conditions followed the protocol (25℃ for 10 min, 37℃ for 120 min, 85℃ for 5 sec, followed by 4℃ indefinitely/ RT complete) and utilized the Gene Amp PCR System 9700 thermal cycler (Applied Biosystems) for cDNA synthesis.

PCR amplification was run utilizing the 7500 fast system (Applied Biosystems, Inc.) that consisted of enzyme activation at 95℃ for 20 sec, followed by 40 cycles of denaturation at 95℃ for 3 sec combined with annealing /extension at 60℃ for 30 sec. All data were analyzed with the ABI 7500 real time PCR system. All TaqMan primer probes used in this study were also purchased from ABI. Data were recorded and analyzed with ABI Sequence Detector Software and graphs visualized with SigmaPlot software. All

data were presented as mean ± standard error of the mean (SEM) and representative of duplicate determinations. Data were normalized to 18S and measured as relative differences using the $2^{-\Delta\Delta CT}$ method as previously described (18,19). Statistical analyses were conducted using SPSS v18. Data were analyzed using analysis of variance, with Tukey's *post-hoc* analysis conducted to assess differences between treatment groups when $p<0.05$.

Nuclear/cytosolic fractionation. Cell were washed with PBS and incubated with ice cold isotonic buffer containing 20 mM Tris, pH 7.4, 125 mM NaCl, 1 mM EGTA, 1 mM EDTA, and 1mM $MgCl_2$, for 10 min on ice. The buffer was supplemented with fleshly prepared 0.1% NP40, and phosphatase/protease inhibitors as described above. Following detergent solubilization, cytosolic fractions were collected from the cell monolayer and clarified by centrifugation (13,000 x *g,* 5 min, 4℃). Intact nuclei were subsequently collected in PBS and gently pelleted by centrifugation (300 x *g,* 3 min, 4℃) Nuclear proteins were extracted in ice cold buffer containing 20 mM Tri (pH 7.4), 1% Triton X, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA supplemented with phosphatase/protease inhibitors and frozen at -80℃. Nuclear fraction were subsequently thawed on ice, sonicated, passes through a 21 gauge needle to shear DNA, and clarified by centrifugation (13,000 x *g,* 10 min, 4℃).

Flow cytometry. Cells were washed with PBS and incubated with Accutase (Millipore, Billerica, MA) for a min to allow cells to detach. Cells were collected into a fresh tube with ice cold staining buffer containing PBS, 2% BSA, and 0.1 % sodium azide. Cells were washed twice by centrifugation (300 x *g,* 5 min). Cells were incubated with Fc receptor blocker (BD Biosciences) on ice for 5 min then incubated with primary

antibodies on ice for 45 min. Cells were then washed twice by centrifugation (300 x *g,* 5 min) and incubated with secondary antibody on ice for 30 min in the dark. Cells were then washed three times as described above and re-suspended in 1 ml of the staining buffer for flow cytometric analysis using Guava easyCyte (Millipore). Data were analyzed using InCyte analysis software (Millipore).

Results

Identification of direct targets involving TNFα-induced inflammatory gene expression. As the GA is well-documented to play an obligatory role in protein secretion, we began these studies by screening for TNFα-induced inflammatory genes expression where mRNA accumulation was both rapid and independent of protein synthesis. This rationale was based on prior observations that TNFα-sensitive gene targets include a subset of genes whose mRNAs accumulate with delayed kinetics consistent with an 'indirect' mechanism requiring synthesis and secretion of TNFα-inducible intermediate transcriptional modulators that regulate gene expression in an autocrine/paracrine manner. Therefore, excluding indirect targets would highlight novel roles for the GA that were independent of protein secretion. We conducted this screen with density-arrested 3T3-L1 preadipocytes (PA) by harvesting total RNA over time following stimulation with 100 pM TNFα. Changes in mRNA abundance were determined by qRT-PCR where data were normalized to 18S ribosomal RNA (rRNA) and expressed as fold differences relative to untreated (UT) controls. Examples of the kinetic screen were illustrated in Fig.4.1 where relative mRNA abundance of two cytokine (p28, EBI3) and two chemokine (CCL2, CCL5) genes were presented where panel A illustrates rapid genes with peak

induction occurring at 4 hrs and panel B showing delayed genes with peak induction at 12 hrs post-TNFα stimulation.

To determine a role for protein synthesis, PAs were pretreated with 5 μM cycloheximide (CHX) for 1 hr prior to stimulating with 100 pM TNFα. Total RNA was harvested at 2 hrs post-stimulation and changes in mRNA abundance determined relative to untreated controls. Total RNA was harvested at a time earlier than peak induction for either group of genes as longer exposures of TNFα in the presence of CHX are well documented to synergistically amplify the effects on cell toxicity (20,21). As depicted in Fig.4.2, inhibition of protein synthesis suppressed TNFα induced expression only for CCL5 whose mRNA was shown above to peak at 12 hrs post-stimulation suggesting the possibility that an indirect mechanism involving secretion of a transcriptional modulator. EBI3 was not examined in this experiment as the 2 hr time point was insufficient for significant mRNA accumulation. In contrast to CCL5, p28 and CCL2 were rapidly induced and independent of protein synthesis suggesting that both genes represented direct targets of TNFα action and unlikely to involve protein secretion as a component of gene expression.

Pharmacological inhibition of GA activity ablates mRNA accumulation of TNFα-induced early and late gene expression. To determine a role for the GA in TNFα-induced gene expression, we initially used the fungal metabolite, brefeldin A (BFA) which is well-documented as having unusual specificity toward blocking guanine nucleotide exchange factors that are required for the recruitment of coat proteins that form the structural lattice that directs the formation and trafficking of vesicles that transport cargo through the GA (22-24). As exposure to BFA has been shown to result in

rapid collapse of the GA, this metabolite has been commonly used as an inhibitor of GA-mediated protein secretion. For these experiments, PAs were pretreated with 1 μM BFA for 1 hr prior to stimulating with 100 pM TNFα. Total RNA was harvested at 2 hrs and 12 hrs post-stimulation and changes in mRNA abundance determined relative to untreated controls. As illustrated in Fig.4.3, BFA ablated TNFα induced expression of both early (A) and delayed (B) genes as determined above. While these results were predicted for delayed genes that were dependent on protein synthesis and, presumably, the secretion of an indirect modulator of TNFα action, the near complete ablation of early TNFα inducible genes was unexpected. As early expressed genes were independent of protein synthesis, these data suggested that the inhibitory effects of BFA likely included mechanisms beyond the well-documented effects on secretory pathways.

To confirm that the unexpected effects of BFA observed above were not gene or phenotype specific, experiments were conducted using undifferentiated PAs as well as differentiated adipocytes (ADs) that display structural and functional differences in phenotype and gene expression. Both cell populations, representing the extremes of the adipocyte lineage, were pretreated in parallel with BFA for 1 hr prior to TNFα stimulation. Total RNA was harvested at 2 hrs post-stimulation and changes in mRNA abundance determined relative to untreated controls for only early expressed gene. As illustrated in Fig.4.4, BFA ablated TNFα-induced expression of cytokines (TNFα, IL-6), chemokines (CCL2, CCL3, CCL20) and other inflammatory modulators (e.g., Cox-2) independent of cell phenotype. To date, we have not identified any TNFα-sensitive inflammatory genes, early or delayed, whose expression is not ablated by BFA suggesting the possibility of a mechanism involving either the receptor or early signaling events that are common to all inflammatory genes that are induced through TNFα action.

Inhibition of GA activity has no effect on mRNA accumulation of LPS or IFNγ induced inflammatory gene expression. As experiments described above demonstrated that most, if not all, TNFα induced gene expression was dependent on GA activity, we next explored the effects of BFA on lipopolysaccharide (LPS) and interferon gamma (IFNγ) induced inflammatory gene expression as both modulators of inflammation are known to activate different cell surface receptors and utilize signaling pathways that are unique from TNFα. For these determinations, PAs were pretreated with BFA for 1 hr prior to LPS (100 ng/ml) or IFNγ (20 ng/ml) stimulation. Total RNA was harvested at 2 hrs post-stimulation and changes in mRNA abundance determined relative to untreated controls. As illustrated in Fig.4.5, LPS (panel A) as well as IFNγ (panel B) induced inflammatory genes displayed no effect of BFA pretreatment supporting the working hypothesis that GA activity was necessary for upstream events regarding TNFα induced gene expression. Furthermore, the absence of any measurable effect of BFA on LPS or INFγ induced genes suggested that the mechanism of action was not likely to include effects of cell toxicity. Lastly, these observations demonstrated that the mechanism responsible for the BFA effect did not include inhibition of general transcriptional machinery (e.g., RNA Pol II).

Nanomolar BFA concentrations reversibly inhibit TNFα-induced inflammatory gene expression. To further support a specific role for BFA on GA activity, we determined the 50% inhibitory concentration (IC50) as well as the reversibility regarding TNFα-induced inflammatory gene expression. Others have shown that nanomolar concentrations of BFA result in dispersal of GA vesicle-associated coat proteins into the cytosol within minutes of exposure and that this process is reversible leading to re-association of coat proteins to GA vesicles as well as reformation of GA

structure and function within hours following removal of BFA from cell media (25-28). To estimate the IC50, PAs were pretreated with increasing concentrations of BFA for 1 hr prior to TNFα stimulation. Total RNA was harvested at 2 hrs post-stimulation and changes in mRNA abundance determined relative to untreated controls. As illustrated in Fig.4.6A, TNFα induced p28 gene expression was suppressed by BFA in a concentration-dependent manner with an estimated IC50 of ~100nM which is consistent with concentrations that have been shown to result in dispersal coat proteins into the cytosol and ensuing collapse of the GA. To determine the reversibility of this effect on gene expression, PAs were pretreated with BFA for 2 hrs prior to a washout period in which BFA was either left on the cells or removed by changing culture media. Cells were then maintained for 2 hrs under conditions of the washout phase (i.e., with or without BFA) prior to TNFα stimulation. Total RNA was harvested at 2 hrs post-stimulation and changes in mRNA abundance determined relative to untreated controls. As illustrated in Fig.4.6B, washout of BFA completely reversed the near complete ablation of TNFα-induced gene expression imposed by BFA. Collectively, these studies suggest that mechanisms by which BFA inhibited TNFα-induced inflammatory gene expression likely include specific effects stemming from vesicular coat protein dispersal leading to structural and functional collapse of the GA.

BFA regulates TNFR cell surface expression. Studies described above demonstrating that BFA inhibited numerous TNFα inducible inflammatory genes with near equal potency suggested the possibility of an upstream mechanism. As BFA had no effect on LPS or IFNγ inducible genes, these observations further highlighted a role for the GA regarding TNFR. To address this premise, we examined TNFR1 and TNFR2 expression on the plasma membrane by flow cytometric analysis over time following

exposure to BFA. As illustrated in Fig.4.7A, both receptors were expressed on the surface of PAs. Furthermore, BFA treatment decreased surface expression of both receptors in a time dependent manner. Quantification of mean fluorescence intensity (Fig.4.7B) further demonstrated a rapid decrease in TNFR2 surface density with a marked reduction observed as early as 15 min following exposure to BFA. While TNFR1 surface expression also decrease with BFA, significant reduction was not observed until 1 hr post-stimulation with no discernible decrease in surface expression noted at 15 min. BFA stimulation for 4 hr reduced surface expression of both receptors to levels that approximated the near ablation of surface density observed following 12 hr BFA exposure. Collectively, these data demonstrated that maintenance of TNFR1 and TNFR2 on the surface of the cell was dependent on GA activity and surface expression was nearly ablated within 4 hr of BFA stimulation suggesting rapid turnover of both receptors on the surface involved a functional GA. The rapidity of receptor turnover was further suggested as both receptors markedly decreased within 1 hr BFA exposure where TNFR2, but not TNFR1, decreased following BFA stimulation for as little as 15 min. As both receptors decreased with time following BFA stimulation, these data further suggested that a decrease in receptor density on the cell surface could account, in part, for the inhibitory effects of BFA on early TNFα-inducible inflammatory gene expression.

BFA regulates TNFα-induced MAPK and NF-κB signaling. As data presented above demonstrated an inhibitory effect of BFA on TNFR surface expression, we next examined the effect of BFA on two major signaling pathways that link receptor activity with TNFα inducible inflammatory gene expression. For these determinations, we examined the phosphorylation states of the mitogen protein kinases (MAPKs) ERK, JNK, and p38 on residues that are well documented as necessary and sufficient for activity as

well as the degradation IκBα as an indirect measure of NF-κB activation. To confirm the specificity of BFA, a structurally distinct pharmacological inhibitor of GA activity, monensin (MNS) was included. As illustrated in Fig.4.8A, TNFα stimulation in the absence of BFA resulted in robust phosphorylation of ERK, JNK and p38 as well as IκBα degradation measured as measured by immunoblotting at 15 min post-TNFα stimulation. While co-stimulation with both TNFα and BFA produced no discernible effects on each signaling pathway, BFA pretreatment for 1 hr or 12 hr suppressed TNFα stimulated MAPK and NF-κB signaling in a manner that closely approximated the effects of BFA of TNFR surface expression as demonstrated above. As TNFR1 surface expression did not decrease until 1 hr following BFA stimulation as shown above, the lack of effect of BFA as a co-treatment on signaling events further supported a role for GA in maintaining TNFR surface expression. The specificity of BFA was further confirmed where MNS produced nearly identical results as those obtained with BFA regarding each signaling pathway (Fig.4.8B).

BFA co-stimulation ablates TNFα-induced gene expression. As we observed that co-stimulation of BFA did not suppress TNFα-activated signaling pathways or TNFR cell surface expression, we next determined the length of BFA pretreatment time necessary for suppression of TNFα induced gene expression. PAs were pretreated with progressively longer pretreatment times ranging from co-stimulation (i.e., 0 min BFA pretreatment) through 1 hr pretreatment that was shown above to ablate TNFα-inducible gene expression. Cells were stimulated with TNFα and changes in mRNA abundance were determined relative to untreated controls. As illustrated in Fig.4.9A, shortening the time of BFA pretreatment to the point of co-stimulation produced no discernible difference from 1 hr pretreatment regarding the inhibitory effect on TNFα inducible TNFα

gene expression. As this observation was unexpected, the results were confirmed with MCP-1 and p28 (Fig.4.9B) where the effects of co-stimulation were identical to those obtained for 1 hr pretreatment. As prolonged BFA pretreatment (i.e., 12 hr) resulted in more dramatic attenuation of inflammatory gene expression compared to BFA co-treatment, these data suggested that the effects of BFA on TNFR cell-surface expression could only account for part of the inhibitory actions attributed to BFA. As BFA co-stimulation dramatically suppressed TNFα-induced gene expression with absolutely no discernible effects on TNFR surface expression or signaling events, these data further highlighted the possibility of a second Golgi-mediated mechanism regulating TNFα-induced inflammatory gene expression downstream of both signaling pathways .

BFA co-stimulation does not suppress signaling magnitude and duration. To confirm that BFA co-stimulation produced no effects on TNFα signaling events, lysates were harvested over time from PAs co-stimulated with BFA and TNFα immunoblotted for phosphorylated ERK, JNK, and p38 as well as IκBα degradation where data were compared to TNFα or BFA stimulation alone. These determinations were different from those presented above where the effects of different BFA pretreatment times on signaling events were measured at a single 15 min time point. As illustrated in Fig.4.10, TNFα rapidly and transiently lead to phosphorylation of all three MAPKs and IκBα degradation with maximum activation observed at 15 min and complete deactivation by 60 min post-TNFα treatment. Moreover, no discernible differences on MAPK or NF-κB signaling magnitude were observed with BFA co-stimulation. While co-stimulation also produced no difference in duration of IκBα degradation, modest differences in duration of phosphorylation (i.e., activity) were noted for each MAPK. While both magnitude and duration of MAPK signaling has been shown

to play a role in biological outcome, the modest differences observed here were unlikely to account for the near complete ablation of TNFα inducible gene expression following BFA co-stimulation.

BFA co-stimulation does not block translocation and phosphorylation of *trans*-acting factors mediating TNFα-induced gene expression. Data presented above demonstrated that BFA co-stimulation significantly suppressed TNFα-induced inflammatory gene expression, but did not inhibit TNFR cell-surface expression or upstream signaling. Therefore, we next examined the effect of BFA on nuclear AP-1 (ATF-2, c-Jun) and NF-κB (p65) transcription factors downstream of MAPK and NF-κB signaling. Cytosolic and nuclear extracts were fractionated from cells at 30 min post-stimulation with BFA and/or TNFα. Optimal cell fractionation was confirmed by immunoblot analysis of nucleoporin and α-tubulin representing nuclear and cytosolic proteins, respectively. Cellular localization was determined for NF-κBp65 as well as phosphorylated ATF-2 and c-Jun as these transcription factors are known to play regulatory roles in TNFα-induced inflammatory gene expression (29-32). As illustrated in Fig.4.11, co-stimulation with BFA did not block TNFα-induced phosphorylation of ATF-2 or c-Jun or blunt nuclear translocation of NF-κBp65. As both measures are indirect indices of activity, these data suggest the mechanism ascribed to the inhibitory effects of BFA co-stimulation on TNFα inducible inflammatory gene expression is likely to involve other modulators of transcription such as co-activator recruitment as opposed to direct effects on mediating transcription factors.

Discussion

We present empirical evidence demonstrating novel roles for the GA in mediating TNFα-induced inflammatory gene expression in adipocytes. First, we show that the Golgi specific inhibitor, BFA ablated TNFα-induced inflammatory gene expression independent of secretory mechanisms requiring protein synthesis and that this inhibition was specific to TNFα. Second, we report that BFA blocked TNFR1 and TNFR2 cell-surface expression contributing to a decrease in MAPK and NF-κB signaling and subsequent gene expression. Third, we report that co-stimulation of BFA with TNFα was sufficient to ablate inflammatory gene expression, independent of TNFR cell-surface expression as well as TNFα signaling pathways. Fourth, we present evidence that TNFα-induced NF-κBp65 and AP-1 nuclear translocation and phosphorylation were not inhibited by BFA co-stimulation. Collectively, data presented in this report suggest that the Golgi plays diverse regulatory roles in mediating TNFα-induced inflammatory gene expression in adipocytes through mechanisms dependent on and independent of TNFR cell-surface expression as well as TNFα signaling.

Early findings demonstrated that TNFα was elevated in AT of obese animals and humans and that genetic and pharmacological ablation of this pleiotropic cytokine improved insulin sensitivity (1,6). Within adipocytes, TNFα binds to TNFR triggering a cascade of events that activate MAPK and NF-κB signaling culminating in IR through inhibitory phosphorylation of IRS-1 in the cytosol as well as activation of inflammatory gene expression in the nucleus (33-37). In other cell types, studies have shown that upon ligand binding, the ligand-receptor complex is internalized in clathrin-coated vesicles that shuttle the ligand to lysosome for degradation and the receptor back to the

membrane via recycling endosomes (13,38). Others have also reported on the dynamic nature of TNFR on the cell surface as membrane-anchored receptors are enzymatically cleaved by the sheddase, TACE and released into the local milieu (39-41). While there is considerable evidence supporting the dynamic turnover of TNFR on the cell surface, it remains unclear what role, if any, the GA plays in TNFR cell surface expression or TNFα activity in adipocytes.

Extensive studies highlight a critical role for the GA in the post-translational modification and distribution of proteins and lipids to the cell surface or to other locations within the cell. Proteins or lipids enter the GA from the ER where they can be modified through glycosylation, phosphorylation, methylation, or acetylation (42-44) prior to being directed to their final destination inside or outside of the cell (45). Indeed, most studies in adipocytes have focused on the role for the GA in trafficking glucose transporter, GLUT4 to the plasma membrane in response to insulin signaling (46,47). While there is growing evidence that the GA plays an additional role in compartmentalizing signaling cascades that originated at the plasma membrane (48), no studies have examined a role for the GA in TNFR trafficking or a role for of the GA in TNFα-induced signaling pathways and inflammatory gene expression in adipocytes.

This report is the first study to show that inhibition of the Golgi complex ablates TNFR cell-surface expression in adipocytes. Emerging evidence has demonstrated that TNFR1 localizes to the GA to serve as a reservoir in regulating cell-surface expression in endothelial and smooth muscle cells (13,15,38). Although we have yet to determine the precise cellular localization of TNFR under basal states as well as under conditions of inflammatory stress, we clearly demonstrate in this report that TNFR1 and TNRF2 are

expressed to some degree on the cell surface functionally coupling TNFα action to MAPK and NF-κB signaling pathways and inflammatory gene expression. Our data also demonstrate that the expression of both receptors on the surface of the cell is a dynamic process highlighting a role the GA in maintaining surface density. While the precise mechanism is currently under investigation, it is plausible that 1) the GA may serve as a reservoir for TNFR1 in adipocytes as has been shown for endothelial cells (13), 2) endocytic recycling of internalized TNFR back to the membrane that may involve essential processing of vesicle cargo by the GA, and 3) surface expression requires de novo TNFR synthesis where the GA may play a role in trafficking of the receptor from the ER to the plasma membrane. As the collapse of the GA with BFA would impinge on either of these possibilities, precise mechanism(s) linking GA activity to TNFR surface expression cannot be concluded at this point.

In addition to regulation of TNFR surface expression, this report further highlights a novel mechanism for the GA in regulating TNFα-induced gene expression that is independent of TNFR surface density or upstream signaling events. Discover of this mechanism was afforded through examination of early response genes whose expression was independent of protein synthesis and, presumably, protein secretion ruling out any role for an intermediate modulator that must the synthesized and secreted from the cell. As BFA co-stimulation ablated TNFα-induced gene expression through mechanisms that did not include changes in surface expression or signaling events from receptor to the nucleus, these data suggest that additional modulators of transcription may be involved. To the point, we hypothesize that a co-activator essential for TNFα-induced gene expression remains sequestered to the GA under basal conditions. Upon TNFα stimulation, the co-activator translocates to the nucleus to regulate inflammatory

gene expression, thereby providing additional control over inappropriate gene expression except when warranted. In support of this hypothesis, emerging evidence demonstrates that various co-activators are localized in the GA and translocate into the nucleus to regulate gene transcription in response to diverse external stimuli (49-51). For instance, the co-activator steroid receptor coactivator (SRC)-3 is sequestered in the lumen of the GA before recruitment with the glucocorticoid receptor (49). Others have shown that multiple co-activators can be sequestered in the GA including those that regulate stress and hormonal transcription (50,51). While elucidation of this premise is underway, this hypothesis may explain the rapid effects of BFA on TNFα-induced inflammatory gene expression that are independent of TNFR surface expression.

In summary, data presented here highlight the important role for the GA in TNFα-mediated gene expression. We show that induction of TNFα-sensitive inflammatory genes was ablated following Golgi collapse with BFA and that these inhibitory actions were specific for TNFα and not generalizable to other inflammatory stimuli. In addition, we report a key role for the Golgi complex in regulating TNFR cell-surface expression as well as TNFα-induced MAPK and NF-κB signaling. Finally, we showed that inhibition of the GA by BFA ablates TNFα-induced inflammatory gene expression through additional mechanisms that are independent of surface expression or signaling events. Based on these observations, we have developed a working model that highlights two mechanistic roles for the GA regarding TNFα-induced inflammatory gene expression (Fig.4.12). First, GA activity is required for maintenance of TNFR surface expression that couples TNFα action to signaling pathways that modulate gene expression, and second, GA activity is required for inflammatory gene expression through processes that are independent of TNFR surface expression as well as TNFα signaling events from receptor to the nucleus,

highlighting the possibility of a GA sequestered co-activator that translocates to the nucleus to impart TNFα actions on gene expression under conditions of inflammatory stress.

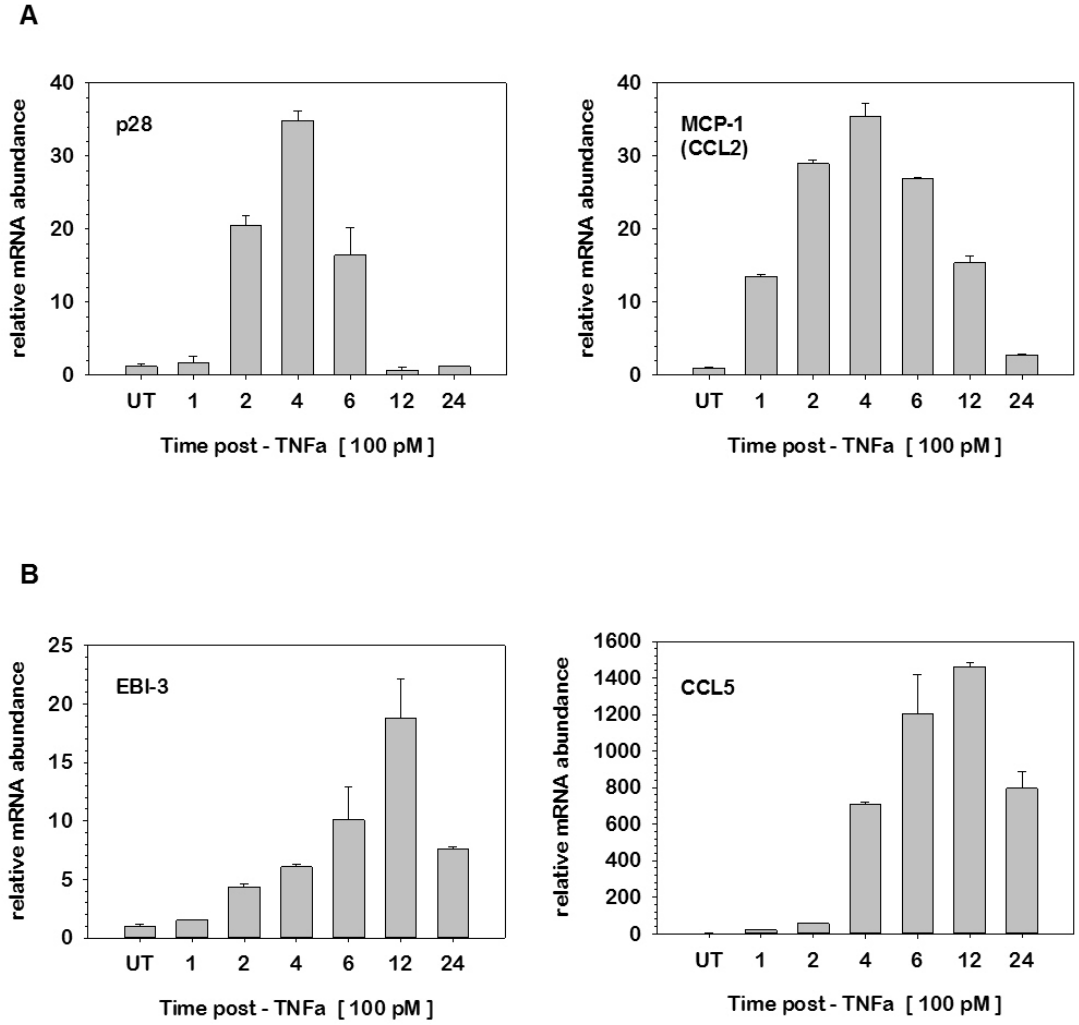

Figure 4.1. TNFα induces inflammatory gene expression with different kinetics. PAs were stimulated with 100 pM TNFα and total RNA was harvested over time post-TNFα prior to mRNA analysis of early genes, p28 and CCL2 (A) and late genes, EBI3 and CCL5 (B) using qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated control (UT).

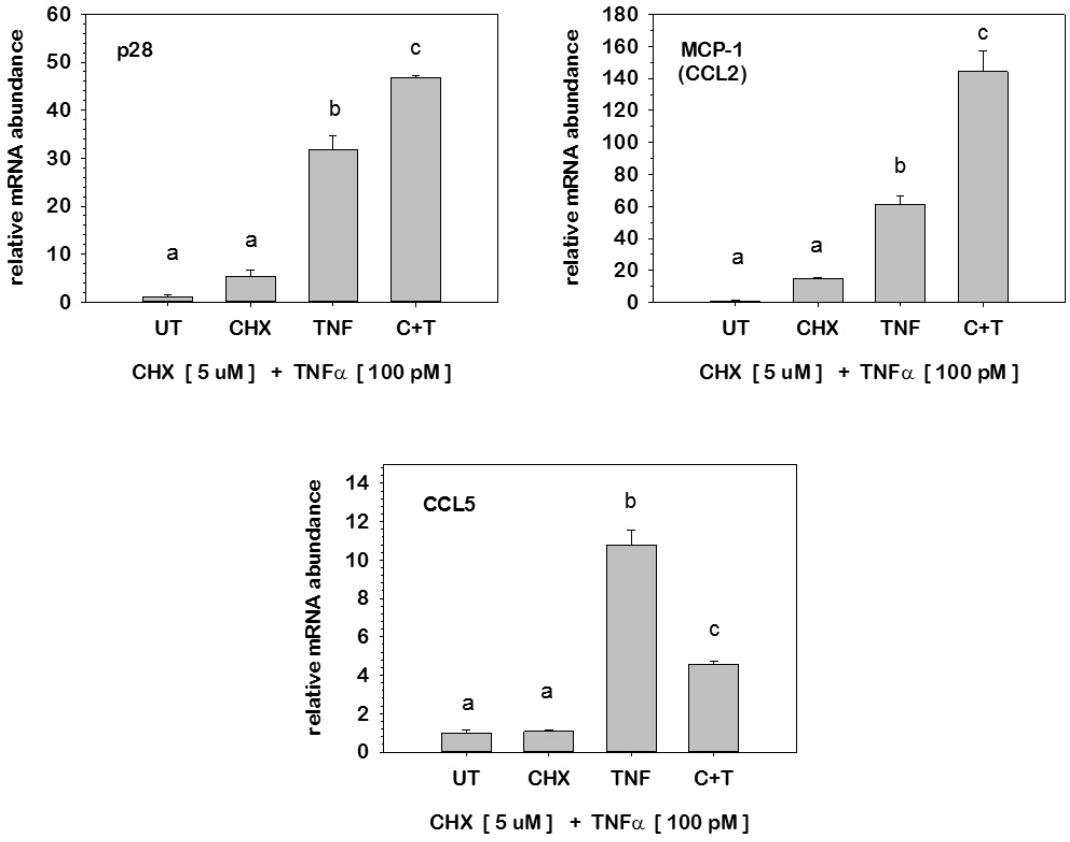

Figure 4.2. TNFα-induced gene expression is independent of de novo protein synthesis. Cells were pretreated with 5 μM CHX for 1 hr prior to 100 pM TNFα stimulation. Total RNA was harvested at 2 hr post-TNFα stimulation and mRNA abundance of p28, CCL2, and CCL5 measured using qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated control (UT). Statistical significance was determined by ANOVA with Tukey's *post-hoc* analysis conducted to assess differences between treatment groups when $p<0.05$.

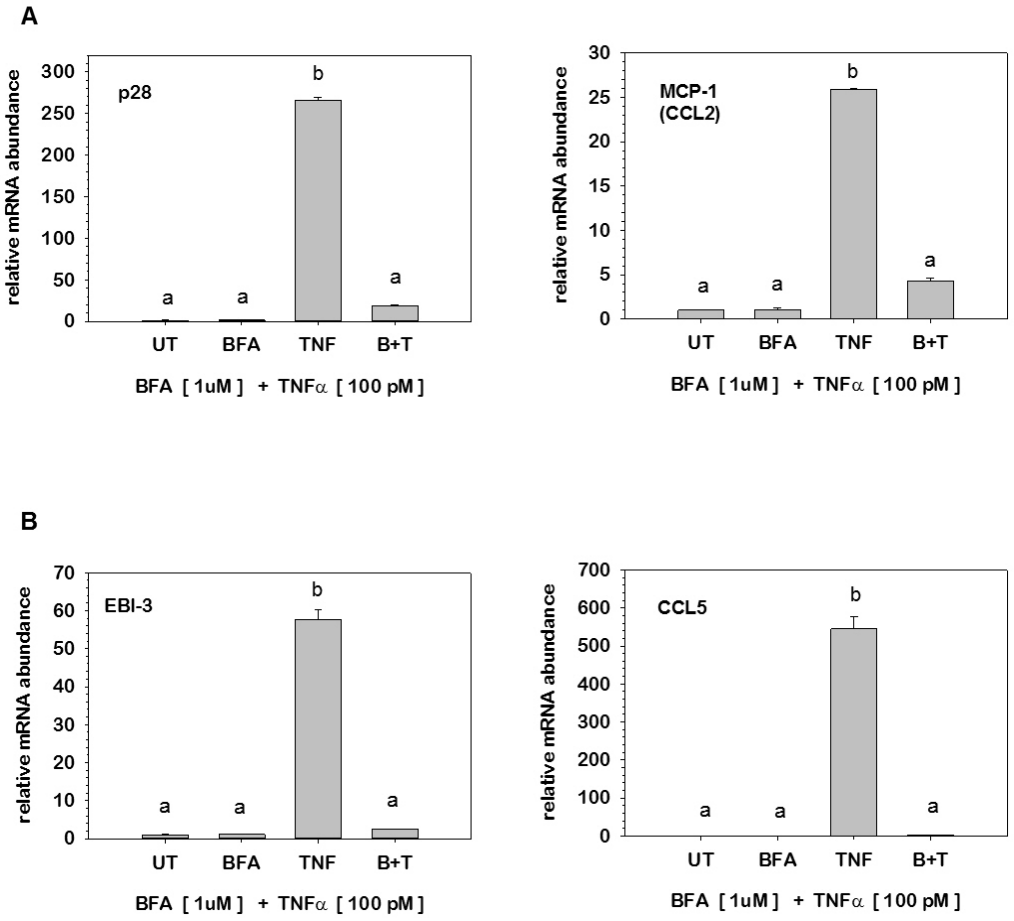

Figure. 4.3. BFA inhibits mRNA accumulation of TNFα-induced early and late gene expression. Cells were pretreated with 1 μM BFA for 1 hr prior to 100 pM TNFα stimulation. Total RNA was harvested at 2 hr (A) or 12 hr (B) post-TNFα stimulation and mRNA abundance of early genes, p28 and CCL2 (A) and late genes, EBI3 and CCL5 (B) measured using qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated control (UT). Statistical significance was determined by ANOVA with Tukey's *post-hoc* analysis conducted to assess differences between treatment groups when $p<0.05$.

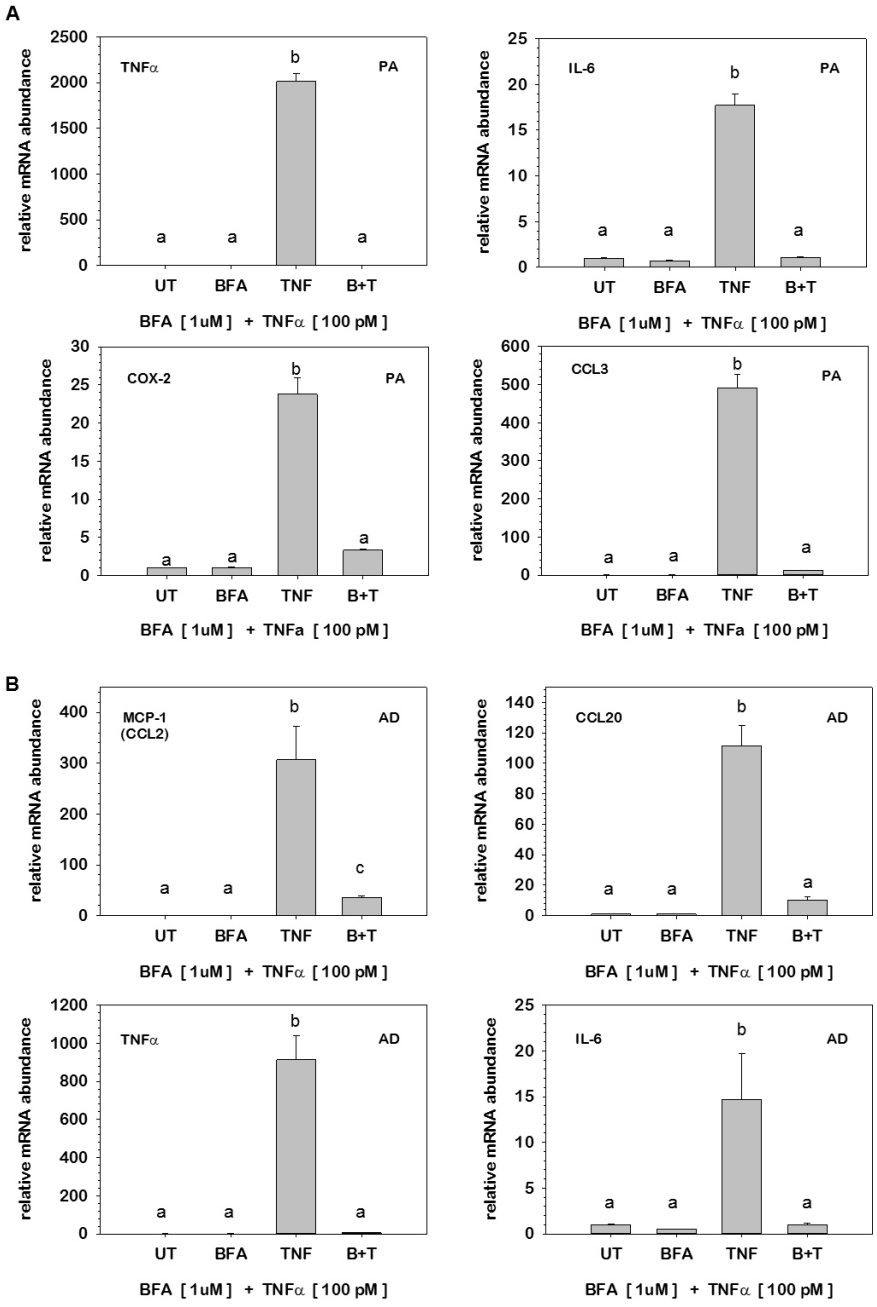

Figure 4.4. BFA inhibits TNFα-induced gene expression in PAs and ADs. PAs (A) and ADs (B) were pretreated for 1 hr in the presence of 1 μM BFA prior to 100 pM TNFα stimulation. Total RNA was harvested at 2 hr post-TNFα stimulation and mRNA abundance measured using qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated control (UT). Statistical significance was determined by ANOVA with Tukey's *post-hoc* analysis conducted to assess differences between treatment groups when $p<0.05$.

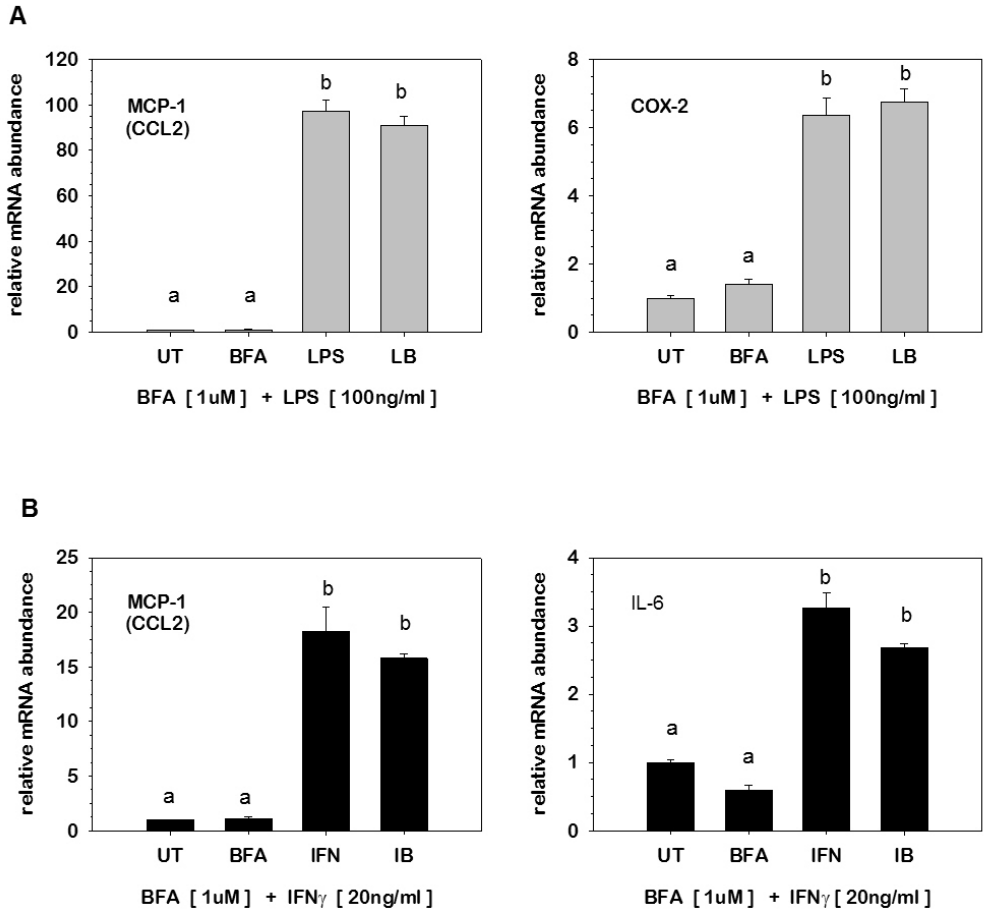

Figure 4.5. BFA does not inhibit LPS- or IFNγ-induced gene expression. PAs were pretreated with BFA for 1 hr prior to 100 ng/ml LPS (A), or 20 ng/ml IFNγ (B) stimulation. Total RNA was collected at 2 hr post-stimulation and mRNA abundance assessed for expression of CCL2, IL-6, and/or COX-2 with qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated control (UT). Statistical significance was determined by ANOVA with Tukey's *post-hoc* analysis conducted to assess differences between treatment groups when $p<0.05$.

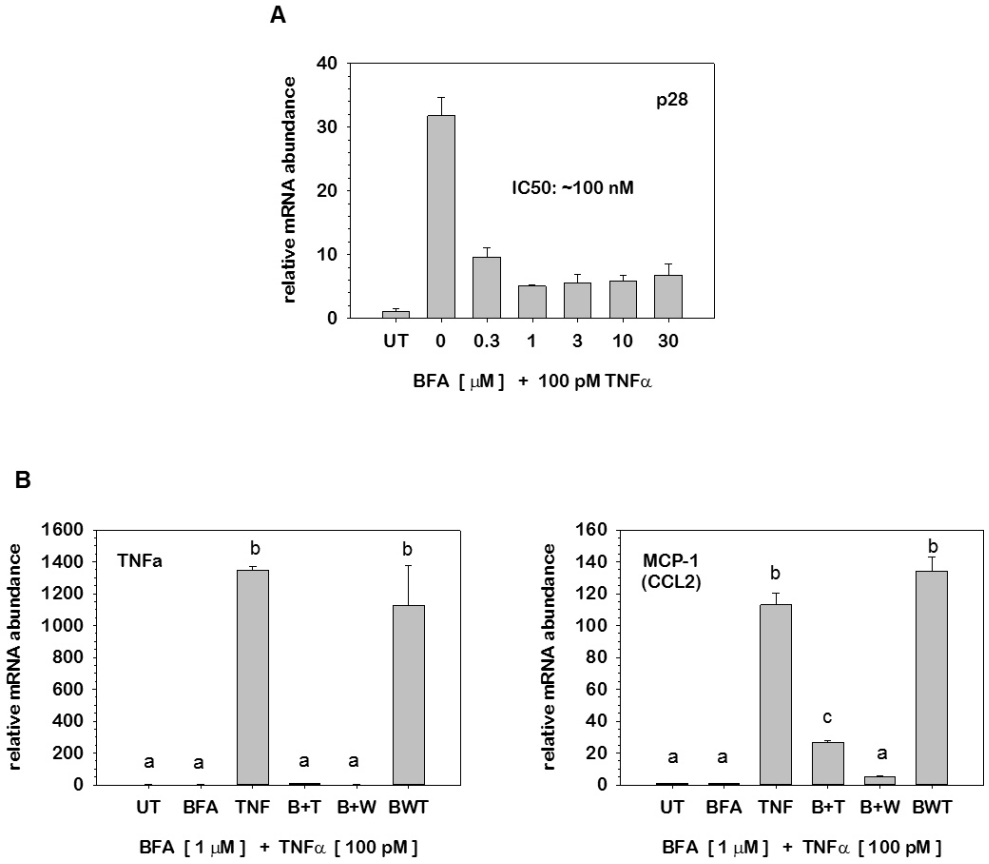

Figure 4.6. Nanomolar BFA concentrations reversibly inhibit TNFα-induced inflammatory gene expression. (A) Cells were pretreated with BFA with varying concentrations for 1 hr prior to 100 pM TNFα stimulation. Total RNA was harvested at 2 hr post-TNFα stimulation and mRNA expression of p28 measured using qRT-PCR. (B) Cells were pretreated with BFA for 2 hr then BFA was removed by changing with fresh culture media for another 2 hr prior to TNFα stimulation. Total RNA was collected at 2 hr post-TNFα treatment and mRNA abundance assessed for expression of CCL2 and TNFα with qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated control (UT). Statistical significance was determined by ANOVA with Tukey's *post-hoc* analysis conducted to assess differences between treatment groups when $p<0.05$.

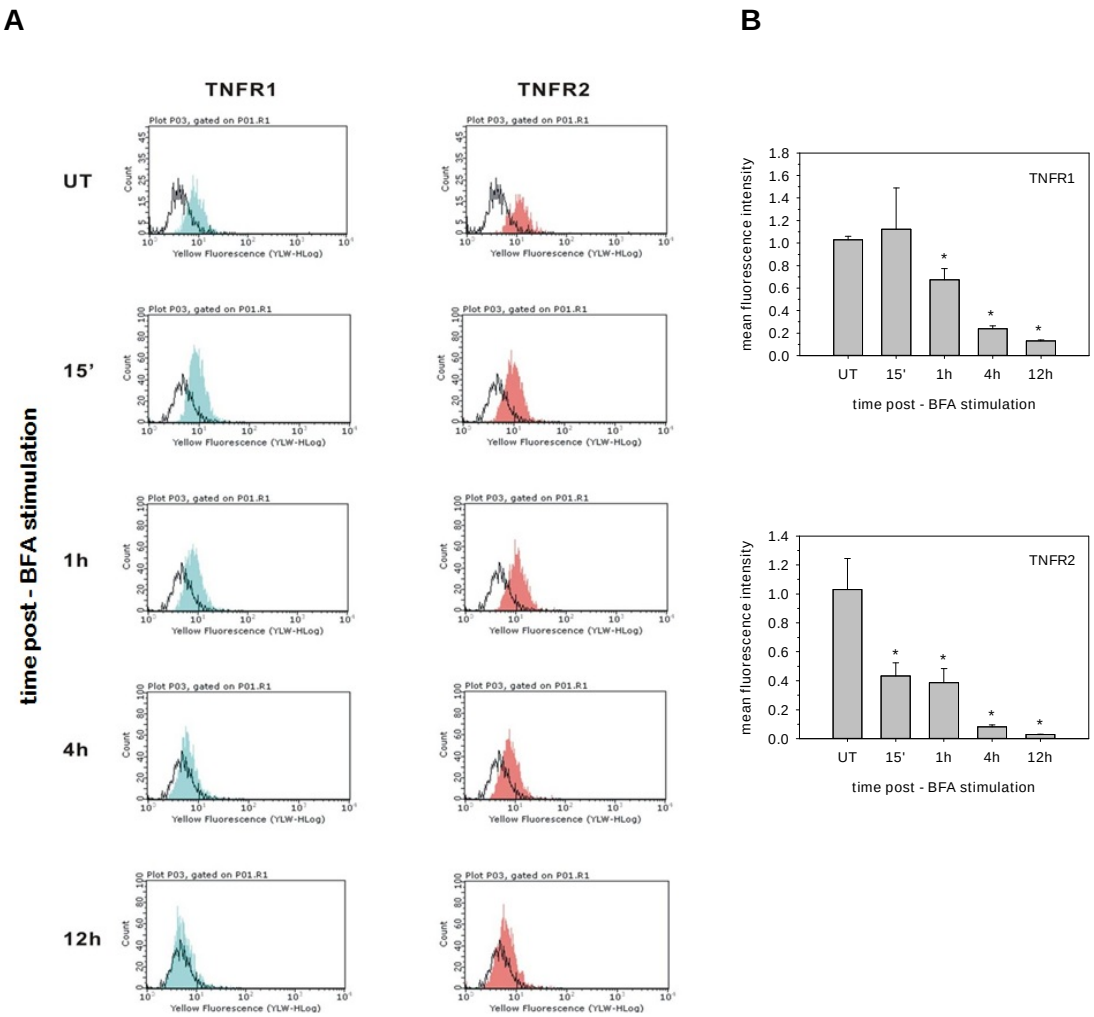

Figure 4.7. BFA decreases expression of TNFR1/2 on cell surface. Cells were treated over time with or without 1 μM of BFA and cell surface TNFR1 and TNFR2 levels were measured by flow cytometry. Cells were harvested, washed, and blocked with purified anti-mouse CD16/CD32 (Fcγ III/II Receptor). Cells were subsequently stained with purified anti-goat TNFR1 and TNFR2 followed by PE-labeled anti-donkey IgG. Staining with the TNFR1 (blue histogram) and TNFR2 (red histogram) antibodies is compared to staining obtained using a purified anti-goat IgG isotype control (open histogram) (A). The mean fluorescence intensity is quantified and plotted in bar graph (B). Statistical significance was determined by ANOVA with Dunnette's *post-hoc* analysis conducted to assess differences from control (UT) when $p<0.05$.

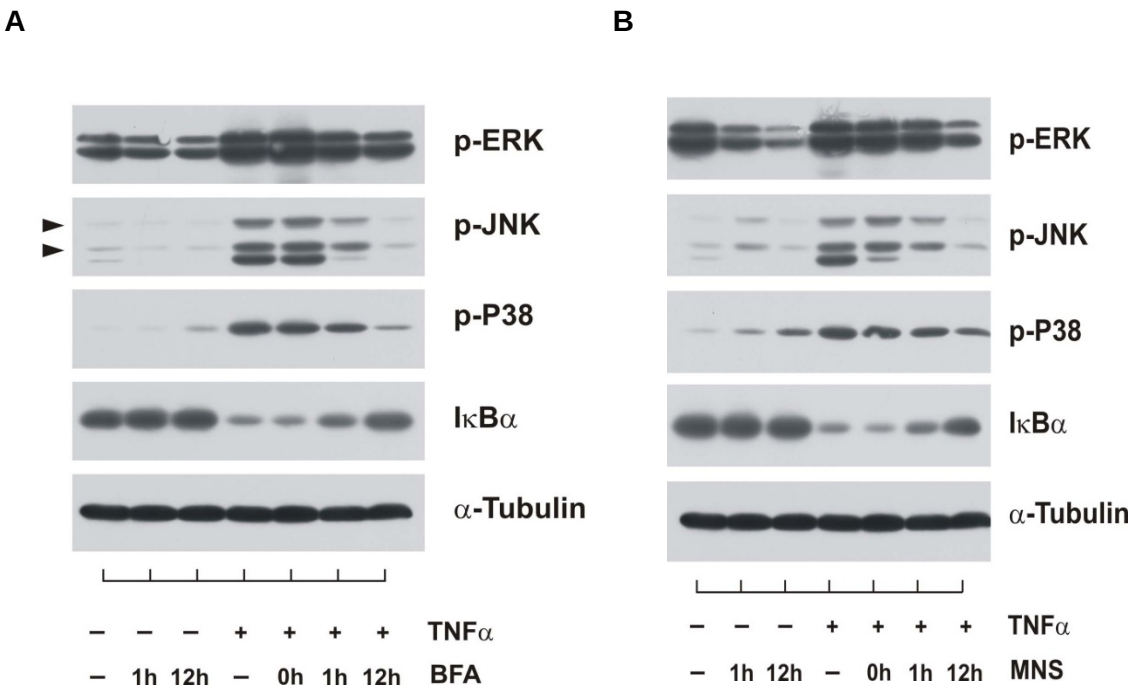

Figure 4.8. Prolonged BFA pretreatment suppresses TNFα-induced changes in signaling events. Density arrested 3T3-L1 PAs were pretreated with or without 1 μM BFA (A) or 30 μM MNS (B) for varying time points prior to 100 pM TNFα stimulation. Cell lysates were collected at 15 min post-TNFα stimulation prior to immunoblot analysis of phosphorylated ERK, JNK, p38, and degradation of IκBα. α-tubulin was used as a loading control.

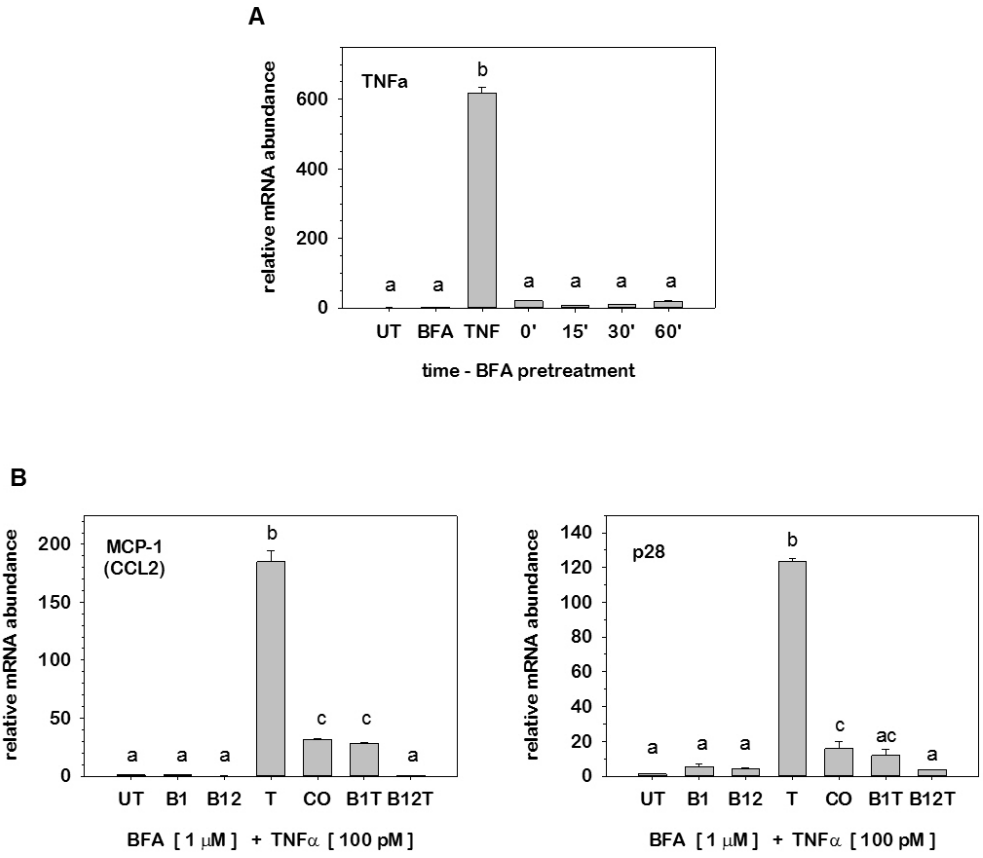

Figure 4.9. BFA co-stimulation blocks TNFα-induced gene expression. Density arrested 3T3-L1 PAs were pretreated with or without 1 μM BFA for varying time points prior to 100 pM TNFα stimulation. Total RNA was collected at 2 hr post-TNFα treatment and mRNA abundance assessed for expression of TNFα (A), CCL2 and p28 (B) with qRT-PCR. Data were normalized to 18S rRNA and expressed relative to untreated control (UT). Statistical significance was determined by ANOVA with Tukey's *post-hoc* analysis conducted to assess differences between treatment groups $p<0.05$.

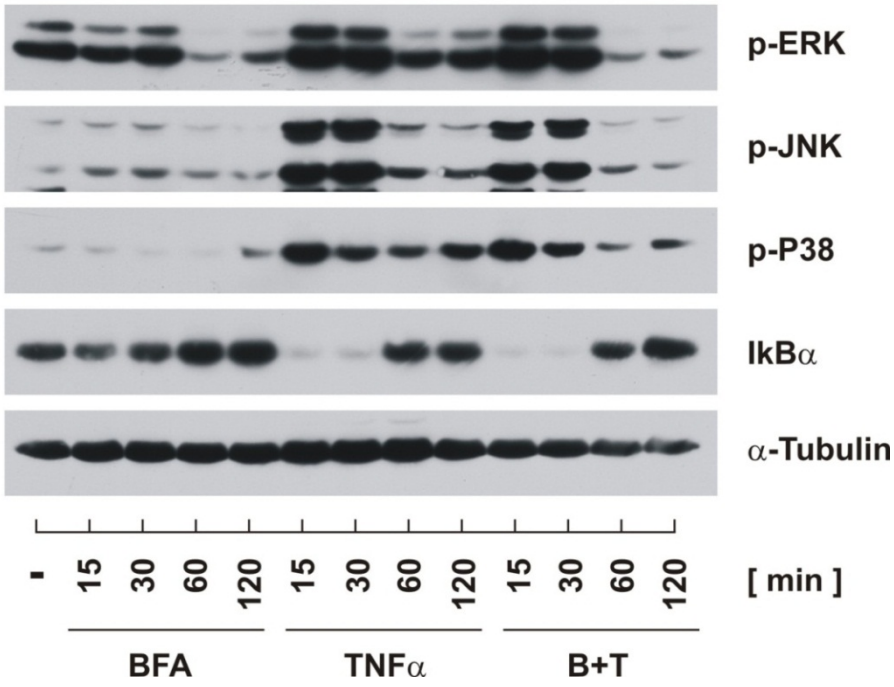

Figure 4.10. BFA co-stimulation does not suppress signaling magnitude and duration. Density arrested 3T3-L1 PAs were co-stimulated with or without 1 μM BFA and 100 pM TNFα. Cell lysates harvested over time following BFA, TNFα, or co-stimulation prior to immunoblot analysis of phosphorylated ERK, JNK, p38 as well as degradation of IκBα. Abundance of α-tubulin served as a loading control.

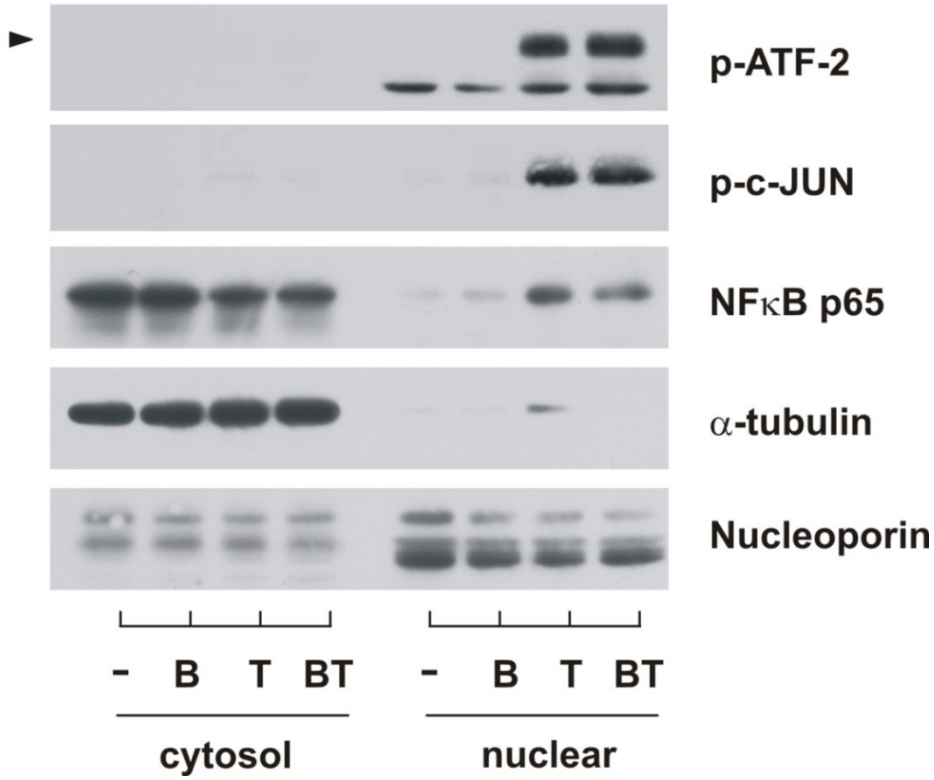

Figure 4.11. BFA co-stimulation does not block TNFα-activated transcription factors in the nucleus. Nuclear and cytosolic extracts were harvested from density arrested 3T3-L1 PAs at 30 min after TNFα stimulation in the absence and presence of BFA (1 μM) and immunoblotted for NF-κBp65, phosphorylated ATF-2, and c-JUN. α-tubulin and nucleoporin were used as markers of cytosolic and nuclear fractions, respectively.

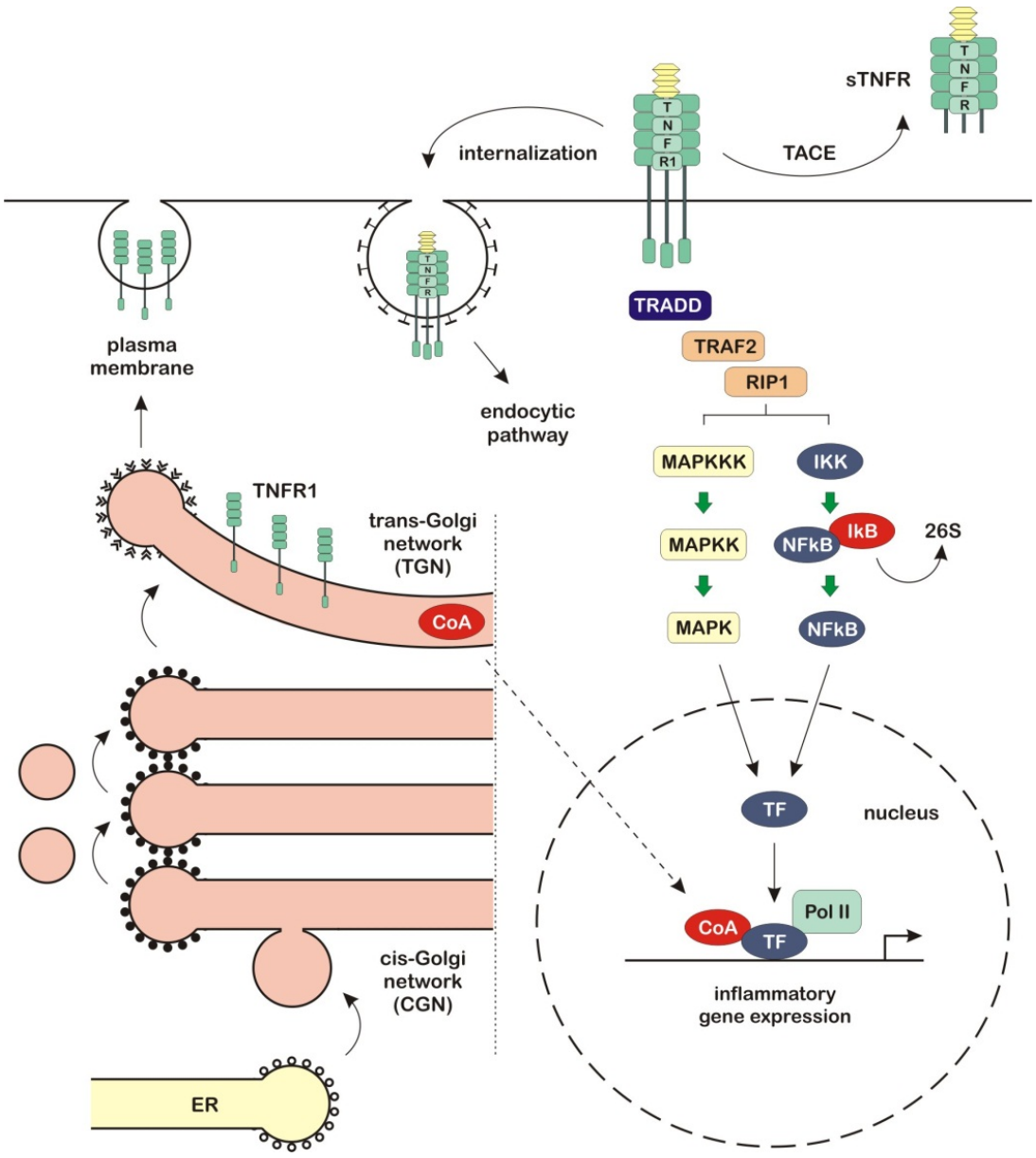

Figure 4.12. Working model for the GA action on TNFα-induced signaling and gene expression. (See text for details)

Reference List

1. Hotamisligil GS, Shargill NS, Spiegelman BM. Adipose expression of tumor necrosis factor-alpha: direct role in obesity-linked insulin resistance. Science. 1993 Jan 1;259:87-91.

2. Wellen KE, Hotamisligil GS. Obesity-induced inflammatory changes in adipose tissue. J Clin Invest. 2003 Dec;112:1785-8.

3. Nieto-Vazquez I, Fernandez-Veledo S, Kramer DK, Vila-Bedmar R, Garcia-Guerra L, Lorenzo M. Insulin resistance associated to obesity: the link TNF-alpha. Arch Physiol Biochem. 2008 Jul;114:183-94.

4. Wellen KE, Hotamisligil GS. Inflammation, stress, and diabetes. J Clin Invest. 2005 May;115:1111-9.

5. Stephens JM, Lee J, Pilch PF. Tumor necrosis factor-alpha-induced insulin resistance in 3T3-L1 adipocytes is accompanied by a loss of insulin receptor substrate-1 and GLUT4 expression without a loss of insulin receptor-mediated signal transduction. J Biol Chem. 1997 Jan 10;272:971-6.

6. Hotamisligil GS, Arner P, Caro JF, Atkinson RL, Spiegelman BM. Increased adipose tissue expression of tumor necrosis factor-alpha in human obesity and insulin resistance. J Clin Invest. 1995 May;95:2409-15.

7. Uysal KT, Wiesbrock SM, Marino MW, Hotamisligil GS. Protection from obesity-induced insulin resistance in mice lacking TNF-alpha function. Nature. 1997 Oct 9;389:610-4.

8. Sethi JK, Xu H, Uysal KT, Wiesbrock SM, Scheja L, Hotamisligil GS. Characterisation of receptor-specific TNFalpha functions in adipocyte cell lines lacking type 1 and 2 TNF receptors. FEBS Lett. 2000 Mar 3;469:77-82.

9. Schneider-Brachert W, Tchikov V, Neumeyer J, Jakob M, Winoto-Morbach S, Held-Feindt J, Heinrich M, Merkel O, Ehrenschwender M, et al. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. Immunity. 2004 Sep;21:415-28.

10. Schutze S, Tchikov V, Schneider-Brachert W. Regulation of TNFR1 and CD95 signalling by receptor compartmentalization. Nat Rev Mol Cell Biol. 2008 Aug;9:655-62.

11. Black RA. Tumor necrosis factor-alpha converting enzyme. Int J Biochem Cell Biol. 2002 Jan;34:1-5.

12. Amour A, Slocombe PM, Webster A, Butler M, Knight CG, Smith BJ, Stephens PE, Shelley C, Hutton M, et al. TNF-alpha converting enzyme (TACE) is inhibited by TIMP-3. FEBS Lett. 1998 Sep 11;435:39-44.

13. Jones SJ, Ledgerwood EC, Prins JB, Galbraith J, Johnson DR, Pober JS, Bradley JR. TNF recruits TRADD to the plasma membrane but not the trans-Golgi network, the principal subcellular location of TNF-R1. J Immunol. 1999 Jan 15;162:1042-8.

14. Storey H, Stewart A, Vandenabeele P, Luzio JP. The p55 tumour necrosis factor receptor TNFR1 contains a trans-Golgi network localization signal in the C-terminal region of its cytoplasmic tail. Biochem J. 2002 Aug 15;366:15-22.

15. Gaeta ML, Johnson DR, Kluger MS, Pober JS. The death domain of tumor necrosis factor receptor 1 is necessary but not sufficient for Golgi retention of the receptor and mediates receptor desensitization. Lab Invest. 2000 Aug;80:1185-94.

16. Djian P, Phillips M, Green H. The activation of specific gene transcription in the adipose conversion of 3T3 cells. J Cell Physiol. 1985 Sep;124:554-6.

17. Morrison RF, Farmer SR. Role of PPARgamma in regulating a cascade expression of cyclin-dependent kinase inhibitors, p18(INK4c) and p21(Waf1/Cip1), during adipogenesis. J Biol Chem. 1999 Jun 11;274:17088-97.

18. Ferguson BS, Nam H, Hopkins RG, Morrison RF. Impact of reference gene selection for target gene normalization on experimental outcome using real-time qRT-PCR in adipocytes. PLoS One. 2010;5:e15208.

19. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods. 2001 Dec;25:402-8.

20. Barbara JA, Smith WB, Gamble JR, Van O, X, Vandenabeele P, Tavernier J, Fiers W, Vadas MA, Lopez AF. Dissociation of TNF-alpha cytotoxic and proinflammatory activities by p55 receptor- and p75 receptor-selective TNF-alpha mutants. EMBO J. 1994 Feb 15;13:843-50.

21. Nio Y, Zighelboim J, Berek J, Bonavida B. Cycloheximide-induced modulation of TNF-mediated cytotoxicity in sensitive and resistant ovarian tumor cells. Cancer Chemother Pharmacol. 1990;26:1-8.

22. Pelham HR. Traffic through the Golgi apparatus. J Cell Biol. 2001 Dec 24;155:1099-101.

23. Marsh BJ, Howell KE. The mammalian Golgi--complex debates. Nat Rev Mol Cell Biol. 2002 Oct;3:789-95.

24. Barr FA. The Golgi apparatus: going round in circles? Trends Cell Biol. 2002 Mar;12:101-4.

25. Lippincott-Schwartz J, Yuan LC, Bonifacino JS, Klausner RD. Rapid redistribution of Golgi proteins into the ER in cells treated with brefeldin A: evidence for membrane cycling from Golgi to ER. Cell. 1989 Mar 10;56:801-13.

26. Lippincott-Schwartz J, Yuan L, Tipper C, Amherdt M, Orci L, Klausner RD. Brefeldin A's effects on endosomes, lysosomes, and the TGN suggest a general mechanism for regulating organelle structure and membrane traffic. Cell. 1991 Nov 1;67:601-16.

27. Fujiwara T, Oda K, Ikehara Y. Dynamic distribution of the Golgi marker thiamine pyrophosphatase is modulated by brefeldin A in rat hepatoma cells. Cell Struct Funct. 1989 Oct;14:605-16.

28. Ulmer JB, Palade GE. Targeting and processing of glycophorins in murine erythroleukemia cells: use of brefeldin A as a perturbant of intracellular traffic. Proc Natl Acad Sci U S A. 1989 Sep;86:6992-6.

29. Ruan H, Hacohen N, Golub TR, Van PL, Lodish HF. Tumor necrosis factor-alpha suppresses adipocyte-specific genes and activates expression of preadipocyte genes in 3T3-L1 adipocytes: nuclear factor-kappaB activation by TNF-alpha is obligatory. Diabetes. 2002 May;51:1319-36.

30. Arkan MC, Hevener AL, Greten FR, Maeda S, Li ZW, Long JM, Wynshaw-Boris A, Poli G, Olefsky J, Karin M. IKK-beta links inflammation to obesity-induced insulin resistance. Nat Med. 2005 Feb;11:191-8.

31. Gil A, Maria AC, Gil-Campos M, Canete R. Altered signalling and gene expression associated with the immune system and the inflammatory response in obesity. Br J Nutr. 2007 Oct;98 Suppl 1:S121-S126.

32. Tilg H, Moschen AR. Inflammatory mechanisms in the regulation of insulin resistance. Mol Med. 2008 Mar;14:222-31.

33. Hotamisligil GS. Inflammation and metabolic disorders. Nature. 2006 Dec 14;444:860-7.

34. Hirosumi J, Tuncman G, Chang L, Gorgun CZ, Uysal KT, Maeda K, Karin M, Hotamisligil GS. A central role for JNK in obesity and insulin resistance. Nature. 2002 Nov 21;420:333-6.

35. Lee YH, Giraud J, Davis RJ, White MF. c-Jun N-terminal kinase (JNK) mediates feedback inhibition of the insulin signaling cascade. J Biol Chem. 2003 Jan 31;278:2896-902.

36. Fujishiro M, Gotoh Y, Katagiri H, Sakoda H, Ogihara T, Anai M, Onishi Y, Ono H, Abe M, et al. Three mitogen-activated protein kinases inhibit insulin signaling by different mechanisms in 3T3-L1 adipocytes. Mol Endocrinol. 2003 Mar;17:487-97.

37. Dandona P, Aljada A, Bandyopadhyay A. Inflammation: the link between insulin resistance, obesity and diabetes. Trends Immunol. 2004 Jan;25:4-7.

38. Wang J, Al-Lamki RS, Zhang H, Kirkiles-Smith N, Gaeta ML, Thiru S, Pober JS, Bradley JR. Histamine antagonizes tumor necrosis factor (TNF) signaling by stimulating TNF receptor shedding from the cell surface and Golgi storage pool. J Biol Chem. 2003 Jun 13;278:21751-60.

39. MacEwan DJ. TNF receptor subtype signalling: differences and cellular consequences. Cell Signal. 2002 Jun;14:477-92.

40. Schneider-Brachert W, Tchikov V, Neumeyer J, Jakob M, Winoto-Morbach S, Held-Feindt J, Heinrich M, Merkel O, Ehrenschwender M, et al. Compartmentalization of TNF receptor 1 signaling: internalized TNF receptosomes as death signaling vesicles. Immunity. 2004 Sep;21:415-28.

41. Tchikov V, Bertsch U, Fritsch J, Edelmann B, Schutze S. Subcellular compartmentalization of TNF receptor-1 and CD95 signaling pathways. Eur J Cell Biol. 2011 Jun;90:467-75.

42. Swift LL. Role of the Golgi apparatus in the phosphorylation of apolipoprotein B. J Biol Chem. 1996 Dec 6;271:31491-5.

43. Teasdale RD, Matheson F, Gleeson PA. Post-translational modifications distinguish cell surface from Golgi-retained beta 1,4 galactosyltransferase molecules. Golgi localization involves active retention. Glycobiology. 1994 Dec;4:917-28.

44. Dmitrieff S, Sens P. Golgi apparatus: Homotypic fusion maintains biochemical gradients within the Golgi and improves the accuracy of protein maturation. Int J Biochem Cell Biol. 2012 Jan 28.

45. Anitei M, Hoflack B. Exit from the trans-Golgi network: from molecules to mechanisms. Curr Opin Cell Biol. 2011 Aug;23:443-51.

46. Fujita H, Hatakeyama H, Watanabe TM, Sato M, Higuchi H, Kanzaki M. Identification of three distinct functional sites of insulin-mediated GLUT4 trafficking in adipocytes using quantitative single molecule imaging. Mol Biol Cell. 2010 Aug 1;21:2721-31.

47. Stockli J, Fazakerley DJ, James DE. GLUT4 exocytosis. J Cell Sci. 2011 Dec 15;124:4147-59.

48. Mayinger P. Signaling at the Golgi. Cold Spring Harb Perspect Biol. 2011 May;3.

49. Grenier J, Trousson A, Chauchereau A, Cartaud J, Schumacher M, Massaad C. Differential recruitment of p160 coactivators by glucocorticoid receptor between Schwann cells and astrocytes. Mol Endocrinol. 2006 Feb;20:254-67.

50. Mori K, Kato H. A putative nuclear receptor coactivator (TMF/ARA160) associates with hbrm/hSNF2 alpha and BRG-1/hSNF2 beta and localizes in the Golgi apparatus. FEBS Lett. 2002 Jun 5;520:127-32.

51. Chen Y, Chen PL, Chen CF, Sharp ZD, Lee WH. Thyroid hormone, T3-dependent phosphorylation and translocation of Trip230 from the Golgi complex to the nucleus. Proc Natl Acad Sci U S A. 1999 Apr 13;96:4443-8.

CHAPTER V

EPILOGUE

This dissertation established novel mechanisms regulating inflammatory gene expression in adipocytes. In addition, findings from the previous chapters along with other observations generated questions for future investigation. These preliminary findings and potential areas of focus will be discussed in detail in this chapter.

Data presented in this dissertation represent empirical evidence demonstrating novel mechanisms involved in adipose tissue (AT) inflammation that potentially link obesity with metabolic diseases. Findings presented in Chapter II collectively demonstrated that interleukin (IL)-12 family cytokines were abundantly expressed in AT and divergently regulated under conditions of genetic and diet-induced obesity that is associated with AT inflammation and insulin resistance (IR). Moreover, we show that IL-27 is secreted from adipocytes and can function as an anti- or pro-inflammatory cytokine based on environmental cues. As we found unique function and regulation of IL-27 from Chapter II, we next examine the regulation of IL-27 in response to inflammatory stress in adipocytes. Data presented in Chapter III highlighted a novel role for histone deacetylases (HDACs) involving divergent mechanisms regulating IL-27 subunits p28 and EBI3 in response to inflammation and adipocyte differentiation. These findings highlight epigenetic alterations on histone as critical marks in the regulation of p28 gene expression, suggesting that HDACs mediate the divergent expression patterns observed

with IL-27. Finally, findings from Chapter IV demonstrated a novel regulatory role for the Golgi apparatus (GA) in regulating tumor necrosis factor alpha receptor (TNFR) cell-surface expression and subsequently TNFα-induced signaling and gene expression in adipocytes. Collectively, data presented in this dissertation provide new mechanisms that have never been studied regarding inflammatory gene expression in adipocytes that potentially link obesity with metabolic inflammatory diseases.

Data presented in this dissertation highlight two IL-27 subunits that were differentially regulated during inflammatory stress and adipoctye differentiation (Ch.2 & 3). Therefore, we further examined how adipocyte differentiation regulates IL-27 expression. Our preliminary data showed that p28 was markedly up-regulated during the course of adipocyte differentiation with peak induction (day4) corresponding to peroxisome proliferator activated receptor γ (PPARγ) activation. PPARγ is a well-established nuclear transcription factor that is necessary and sufficient for adipocyte differentiation (1,2). Preliminary findings from our lab further demonstrated that induction of p28 required the same activators of adipocyte differentiation as PPARγ. This was readily apparent from our 3T3-L1 cell line stably expressing a dominant-negative isoform for CCAAT enhancer binding protein β (C/EBPβ) (L1-LIP), as p28 gene expression was lost in response to adipocyte differentiation. C/EBPβ activity is necessary for PPARγ induction and activation, and therefore is a key regulator during early adipocyte differentiation (3). Thus, similar to p28 gene expression, PPARγ gene and protein expression was significantly attenuated. These data suggested a potential role for both PPARγ and C/EBPβ in the regulation of p28 gene expression. Consistent with this hypothesis, others have shown PPAR and C/EBP binding site within the p28 promoter (4,5).

In contrast to p28, we also determined that EBI3 was dramatically decreased during adipogenesis (Ch.2 & 3). Findings from our lab determine that the decrease in EBI3 gene expression occurred during early adipocyte differentiation (<12 hrs). As initiation of 3T3-L1 adipocyte differentiation requires a hormonal cocktail that consists of methyl-isobutylxathine (MIX), insulin, and dexamethasone, we can subsequently tease out signaling pathways regulating early gene expression by isolating individual components of this cocktail. Our findings demonstrate that MIX was primarily responsible for the suppression of EBI3. Extensive studies have shown that MIX regulates the cAMP/PKA signaling pathway and its downstream transcription factors, cAMP-response element binding protein (CREB) and C/EBPβ (6,7). Subsequently, we observe that treatment with the cAMP/PKA inhibitor, H-89 rescued EBI3 gene repression during early adipogenesis, suggesting a regulatory role for cAMP/PKA in EBI3 mRNA expression. As the progression of obesity involves expansion of AT mass through an increase in size and number of adipocytes as well as recruitment of macrophages that contribute to inflammation, future examination of a role for adipocyte differentiation in regulating inflammatory gene expression in adipocytes may highlight obesity-mediated inflammation in AT.

Observations presented above highlight future studies involving inflammatory gene regulation during adipogenesis. In this dissertation, we also demonstrate a regulatory role for HDACs that is gene-type and cell-type specific, potentially contributing to divergent regulation of IL-27 in response to stress (Ch.3). Preliminary findings from our lab demonstrate that HDACs play a critical role in adipocyte inflammation as HDAC inhibitors attenuated expression of several TNFα-induced inflammatory genes that are largely shown to be involved in obesity-associated inflammation. Therefore, we raise

many questions from the previous chapter with regard to involvement of HDACs in adipocyte inflammation. For example, 1) do specific HDACs are required for TNFα-induced inflammatory gene expression, 2) are there any cell-type specific HDACs regulating adipocyte inflammation based on degree of adipocyte differentiation, and 3) what mechanisms are involved in these processes? Anti-inflammatory actions have been ascribed to HDAC inhibitors in vitro and in vivo, with most studies focused on class I and class II HDACs as potential therapeutic targets (8,9). While many of these anti-inflammatory properties appear to be driven by epigenetic regulation of gene transcription (10), HDACs can deacetylate many lysine residues on non-histone proteins (11). It has been shown that HDACs are highly involved in adipocyte function (12-15), while others demonstrate that HDACs play a crucial role in TNFα-mediated transcriptional activity via NF-κB dependent inflammatory gene expression (16,17). Our lab previously observed an anti-inflammatory role for HDAC inhibitors on TNFα-induced inflammatory gene expression in adipocytes. Therefore, further investigation into the role of HDACs in the context of adipocyte inflammation will increase our understanding of the epigenetic control link obesity with metabolic inflammatory diseases.

Finally, emerging evidence has demonstrated that the GA is a critical organelle involved in modifying and packaging proteins in route to their final destination (18). Moreover, diverse signaling factors are sequestered to the Golgi and control signaling cascades in response to external cues (19). Lastly, evidence demonstrates that the Golgi complex regulates cell-surface receptor expression and thus serves as an important organelle for intracellular signaling. For example, several components of the Ras and protein kinase A pathways reside at the Golgi, suggesting an important role for the GA in compartmentalizing signal transduction pathways (18,20). However, little is

known about the regulatory role for the GA in inflammatory signaling and gene expression in adipocytes. Data highlighted in Chapter IV demonstrate a novel regulatory role for the GA in TNFα-mediated inflammatory signaling and gene expression in adipocytes, as inhibition of GA function resulted in suppression of TNFα-induced gene expression as well as TNFR cell-surface expression and upstream signaling. However, suppression of inflammatory genes preceded TNFR cell-surface suppression as well as TNFα-induced signaling in response to the Golgi inhibition. These findings suggest an unreported role for the GA in inflammatory gene expression that is independent of TNFR surface density or signaling pathways that mediate TNFα action. Thus, it could explain that the GA contains co-factors that are subsequently released in response to TNFα to regulate early gene transcription. To further understand this, it is crucial to identify TNFα-specific co-regulators that are localized to the Golgi and utilize gain- and loss-of-function studies to elucidate a functional role for these co-factors on TNFα-mediated inflammation. These studies would increase our understanding of a role for the GA on TNFα-mediated signaling pathways and gene expression in adipocytes.

Data presented in Chapters II-IV significantly contribute to the limited body of knowledge regarding inflammation in adipocytes. Use of the murine 3T3-L1 cell line provides an excellent model to study adipocyte inflammation, as this cell line does not contain other cell types (e.g., macrophages). This condition allows us to delineate inflammatory signaling events in a homogenous population of PAs or ADs. Furthermore, our findings lead to ample questions for future studies related to novel mechanisms involved in adipocyte inflammation through regulation of multiple pro-inflammatory genes that contribute to inflammation and IR. It is expected that continued examination of novel adipokines as well as a role for HDACs and the GA in inflammatory signaling and gene

expression will provide researchers with a better understanding of adipocyte biology involved in obesity, inflammation, and IR.

Reference List

1. Morrison RF, Farmer SR. Insights into the transcriptional control of adipocyte differentiation. J Cell Biochem. 1999;Suppl 32-33:59-67.

2. Morrison RF, Farmer SR. Hormonal signaling and transcriptional control of adipocyte differentiation. J Nutr. 2000 Dec;130:3116S-21S.

3. Hamm JK, Park BH, Farmer SR. A role for C/EBPbeta in regulating peroxisome proliferator-activated receptor gamma activity during adipogenesis in 3T3-L1 preadipocytes. J Biol Chem. 2001 May 25;276:18464-71.

4. Zhang J, Qian X, Ning H, Yang J, Xiong H, Liu J. Activation of IL-27 p28 gene transcription by interferon regulatory factor 8 in cooperation with interferon regulatory factor 1. J Biol Chem. 2010 Jul 9;285:21269-81.

5. Molle C, Nguyen M, Flamand V, Renneson J, Trottein F, De WD, Willems F, Goldman M, Goriely S. IL-27 synthesis induced by TLR ligation critically depends on IFN regulatory factor 3. J Immunol. 2007 Jun 15;178:7607-15.

6. Zhang JW, Tang QQ, Vinson C, Lane MD. Dominant-negative C/EBP disrupts mitotic clonal expansion and differentiation of 3T3-L1 preadipocytes. Proc Natl Acad Sci U S A. 2004 Jan 6;101:43-7.

7. Lane MD, Tang QQ, Jiang MS. Role of the CCAAT enhancer binding proteins (C/EBPs) in adipocyte differentiation. Biochem Biophys Res Commun. 1999 Dec 29;266:677-83.

8. Han SB, Lee JK. Anti-inflammatory effect of Trichostatin-A on murine bone marrow-derived macrophages. Arch Pharm Res. 2009 Apr;32:613-24.

9. Zhou X, Hua X, Ding X, Bian Y, Wang X. Trichostatin Differentially Regulates Th1 and Th2 Responses and Alleviates Rheumatoid Arthritis in Mice. J Clin Immunol. 2011 Feb 9.

10. Mahlknecht U, Will J, Varin A, Hoelzer D, Herbein G. Histone deacetylase 3, a class I histone deacetylase, suppresses MAPK11-mediated activating transcription factor-2 activation and represses TNF gene expression. J Immunol. 2004 Sep 15;173:3979-90.

11. Chen LF, Greene WC. Shaping the nuclear action of NF-kappaB. Nat Rev Mol Cell Biol. 2004 May;5:392-401.

12. Chatterjee TK, Idelman G, Blanco V, Blomkalns AL, Piegore MG, Jr., Weintraub DS, Kumar S, Rajsheker S, Manka D, et al. Histone deacetylase 9 is a negative regulator of adipogenic differentiation. J Biol Chem. 2011 Aug 5;286:27836-47.

13. Yoo EJ, Chung JJ, Choe SS, Kim KH, Kim JB. Down-regulation of histone deacetylases stimulates adipocyte differentiation. J Biol Chem. 2006 Mar 10;281:6608-15.

14. Kim SN, Choi HY, Kim YK. Regulation of adipocyte differentiation by histone deacetylase inhibitors. Arch Pharm Res. 2009 Apr;32:535-41.

15. Haberland M, Carrer M, Mokalled MH, Montgomery RL, Olson EN. Redundant control of adipogenesis by histone deacetylases 1 and 2. J Biol Chem. 2010 May 7;285:14663-70.

16. Inoue K, Kobayashi M, Yano K, Miura M, Izumi A, Mataki C, Doi T, Hamakubo T, Reid PC, et al. Histone deacetylase inhibitor reduces monocyte adhesion to endothelium through the suppression of vascular cell adhesion molecule-1 expression. Arterioscler Thromb Vasc Biol. 2006 Dec;26:2652-9.

17. Grabiec AM, Korchynskyi O, Tak PP, Reedquist KA. Histone deacetylase inhibitors suppress rheumatoid arthritis fibroblast-like synoviocyte and macrophage IL-6 production by accelerating mRNA decay. Ann Rheum Dis. 2012 Mar;71:424-31.

18. Sallese M, Giannotta M, Luini A. Coordination of the secretory compartments via inter-organelle signalling. Semin Cell Dev Biol. 2009 Sep;20:801-9.

19. Mayinger P. Signaling at the Golgi. Cold Spring Harb Perspect Biol. 2011 May;3.

20. Quatela SE, Philips MR. Ras signaling on the Golgi. Curr Opin Cell Biol. 2006 Apr;18:162-7.